UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: FISIOLOGIA

HELENA TREVISAN SCHROEDER

AVALIAÇÃO DA RESPOSTA DA VIA DE CHOQUE TÉRMICO NO DESENVOLVIMENTO DA RESISTÊNCIA À INSULINA E OBESIDADE

PORTO ALEGRE, 2024

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Fisiologia, do Departamento de Fisiologia do Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), como requisito para obtenção do grau de Doutora em Fisiologia.
Orientador: Prof. Dr. Paulo Ivo Homem de Bittencourt Júnior

PORTO ALEGRE, 2024

AGRADECIMENTOS

Sou grata á minha família e à nossa história de crescimento e amadurecimento. Obrigada aos meus: pais, Célia e Hermes, cada um, à sua maneira, contribuíram para que eu chegasse até esse momento; tio Paulo e vó Almira, que me ensinaram tanto; Artur, meu irmão, por quem eu agradeço por ser quem é epor quem torço muito para que realize todas suas aspirações; Carlos sempre meu grande companheiro, que contribuiu com apoio emocional e técnico para que essa tese fosse desenvolvida; dona Marlene, Ana e Lúcio, pessoas maravilhosas que admiro muito. Obrigada por tudo.

Meu profundo agradecimento à Maria Inês, a Inesinha, que me acompanhou e incentivou nos últimos dez anos. Além de auxiliar na parte prática do trabalho, também foi responsável por montar e organizar toda a mudança do nosso laboratório para o novo prédio. Aprendi muito com ela sobre organização e dedicação ao trabalho. Obrigada por toda a amizade.

Tenho muito a agradecer ao meu professor orientador prof. Paulo Ivo, que me acompanha desde o inicio da graduação e me oportunizou fazer parte do seu grupo de pesquisa. O prof é uma pessoa incrivelmente humilde e que me ensinou muito sobre diferentes assuntos. Sou muito grata pelo tempo que convivi com o senhor.

À minha equipe oficial Marcela, Victor e Cris, que me auxiliaram em diferentes estágios de execução dos trabalhos. A todos os componentes do nosso grupo de pesquisa, especialmente a Layane, Mariana, Henrique e Dona Ritinha, foi ótimo conhecer vocês.

Agradeço aos professores Mauricio Krause e Patrícia Bock, pela honra de poder compartilhar de seus conhecimentos e me proporcionarem tantas oportunidades científicas, pelas quais sou muito grata.

Aos meus amigos de trabalho e parceiros de pesquisa, que não estavam mais em nosso laboratório durante meu doutorado, mas que contribuíramenormementedurante a minha formação acadêmica, em especial a Aline, Cinthia, Everton Cristian, Gisele, Graziella, Ítalo, Jorge Roberto, Maciel, Leandro, Sofia e Rossana.

Gostaria de estender meus agradecimentos a todos os funcionários do ICBS/UFRGS, por toda a estrutura, sem a qual não seria possível o desenvolvimento do meu trabalho. Bem como sou grata ao PPG Fisiologia UFRGS pelo curso do qual tive a oportunidade de fazer parte. Também gostaria de deixar um agradecimento especial à instituição (UFRGS), e a todas as pessoas que direta ou indiretamente contribuíram para a realização da minha pesquisa.

FINANCIAMENTO: Este estudo foi financiado em parte com recursos públicos provenientes do CNPq e da CAPES.

RESUMO

A resposta de choque térmico (HSR) é um mecanismo altamente conservado que esta relacionada à manutenção da proteostase, do metabolismo energético e da resolução da inflamação. O principal efeito dessa resposta é o aumento da expressão da proteína de choque térmico de 70 kDa (HSP70). O bloqueio da sua expressão parece estar diretamente ligado a progressão de doenças inflamatórias crônicas. Assim, a presente tese teve como objetivos: a) descrever, por meio de três revisões narrativas, o histórico do estudo da HSR na proteostase e metabolismo energético; o papel da HSR na resolução da inflamação e no desenvolvimento de doenças crônico-inflamatórias; métodos de indução e aferição da HSR. E b) investigar, a partir de dois trabalhos experimentais originais, se a capacidade de disparo da HSR está correlacionada ao nível de sensibilidade à insulina, podendo ser medida de maneira representativa a partir do desafio térmico das células imunológicas do sangue. Bem como, se a terapia hipertérmica é capaz de melhorar os índices de composição corporal e glicêmicos alterados pela indução da obesidade por dieta hiperlipídica. Para os estudos experimentais foram utilizados camundongos C57BL/6J machos submetidos à dieta padrão (NC) ou hiperlipídica (HFD, 60 % da kcal em lipídios) desde o desmame. Acompanhados por 1, 4, 8, 10, 14, 18 e 22 semanas de dieta (capítulo IV), mostrando que a variação da expressão de HSP70 (42 °C - 37 °C) se ajusta a uma regressão logística de 5 parâmetros com a progressão da resistência à insulina e prediz o aparecimento de diabetes tipo 2 nestes animais, oferecendo uma ferramenta para avaliar a disfunção da HSR na inflamação relacionada à obesidade. E animais também tratados com dieta foram submetidos a partir da 14ª semana de dieta a uma sessão semanal de terapia hipertérmica (HT, por 15 min à 41,0 - 41,7 °C) ou não (SHAM, mantidos a temperatura ambiente) por mais quatro ou oito semanas (capítulo V). O HT foi capaz de reduzir o ganho de peso nos animais tratados, melhorando a glicemia de jejum e induzindo expressão de HSP72 no músculo gastrocnêmio.

ABSTRACT

The heat shock response (HSR) is a highly conserved mechanism that is related to the maintenance of proteostasis, energy metabolism and resolution of inflammation. The main effect of this response is the increase in the expression of the 70 kDa heat shock protein (HSP70), and blocking its expression appears to be directly linked to the progression of chronic inflammatory diseases. Thus, the present thesis aimed to: a) describe, through three narrative reviews, the history of the study of HSR in proteostasis and energy metabolism; the role of HSR in the resolution of inflammation and the development of chronic-inflammatory diseases; HSR induction and measurement methods. And b) investigate, based on two original experimental studies, whether the ability to trigger the HSR is correlated to the level of insulin sensitivity, and can be measured in a representative way based on the thermal challenge of immune cells in the blood. As well as whether hyperthermic therapy is capable of improving body composition and glycemic indexes altered by the induction of obesity by a high-fat diet. For experimental studies, male C57BL/6J mice were used on a standard (NC) or high-fat diet (HFD, 60% of kcal in lipids) since weaning. Followed by 1, 4, 8, 10, 14, 18 and 22 weeks of diet (chapter IV), showing that the variation in HSP70 expression (42 °C - 37 °C) fits a 5-parameter logistic regression with the progression of insulin resistance and predicts the onset of type 2 diabetes in these animals, offering a tool to assess HSR dysfunction in obesity-related inflammation. And animals also treated with diet were subjected from the 14th week of diet to a weekly session of hyperthermic therapy (HT, for 15 min at 41.0 - 41.7 °C) or not (SHAM, kept at room temperature) for another four or eight weeks (chapter V). HT is capable of reducing weight gain in treated animals, improving fasting glycemia and inducing HSP72 expression in the gastrocnemius muscle.

LISTA DE ABREVIATURAS E SIGLAS

DMT2: diabetes *mellitus* do tipo 2

ELISA: *enzyme linked immuno sorbent assay,* ensaio de imunoabsorção enzimática

HOMA: *homeostatic model assessment,* modelo de avaliação da homeostase

HSF-1: *heat shock factor-1,* fator de choque térmico 1

HSP: *heat shockprotein,* proteína de choque térmico

HSP70: proteína de choque térmico de 70 kDa

HSR: *heat shock response,* resposta de choque térmico

IR: resistência à insulina

JNK: proteina c-jun n-terminal quinase

NF-κB: *nuclear factor-kappa B,* fator nuclear kappa B

QUICKI: *quantitative insulin sensitivity check index,* índice quantitativo de verificação da sensibilidade à insulina

SIRT-1: sirtuína 1

UPR: *unfolded protein response*, resposta a proteínas desnoveladas

NLRP3: *NOD-like receptor pyrin domain-containing protein 3*

ER: retículo endoplasmático

LUCA: *last universal common ancestor,* ultimo ancestral comum universal

TA: tecido adiposo

SASP: *senescence-associated secretory phenotype,* fenótipo secretório associado à senescencia

RNA: ácido ribonucléico

mRNA: ácido ribonucléico mensageiro

SUMÁRIO

APRESENTAÇÃO DA TESE

Visando a compilação dos trabalhos desenvolvidos, esta tese está dividida em: a) uma introdução que aborda os tópicos gerais que permeiam e servem de fio condutor dos artigos aqui apresentados (resposta de choque térmico, obesidade, resistência à insulina, inflamação e o tratamento hipertérmico); b) cinco capítulos, contendo o desenvolvimento da tese, apresentados em formato de artigo; c) uma discussão geral relacionando todos os capítulos; d) as conclusões finais da tese.

Os capítulos de I a III apresentam os artigos de revisão narrativa desenvolvidos, que aqui também servem como referencial teórico, tratando da resposta de choque térmico: na proteostase e metabolismo energético (**artigo 1**); na resolução da inflamação e no desenvolvimento de doenças crônico-inflamatórias (**artigo 2**); relacionando aos seus métodos de indução e aferição (**artigo 3**). O capítulo IV refere-se ao primeiro artigo original no qual foi realizado o acompanhamento do desenvolvimento da obesidade, o perfil de sensibilidade à insulina relacionado à capacidade de resposta ao choque térmico em camundongos C57BL/6J no desmame, tratados com dietas padrão ouhiperlipídica por 1, 4, 8, 10, 14, 18 ou 22 semanas (**artigo 4**). O capítulo V descreve os resultados da intervenção com tratamento térmico em animais mantidos por 18 e 22 semanas com dieta hiperlipídica ou padrão (**artigo 5**).

INTRODUÇÃO

Evolutivamente, os organismos precisaram se adaptar ao ambiente e alguns mecanismos fisiológicos foram favorecidos em detrimento de outros, gerando os sistemas homeostáticos que encontramos atualmente. Um alto grau de conservação do mecanismo, significando que ele está presente há mais tempo na cadeia evolutiva, é um forte indício da importância deste para a sobrevivência celular entre as diferentes espécies (Leong; Uesaka; Irie, 2022). Temos como exemplos, de mecanismos altamente conservados, os sistemas envolvidos na manutenção da proteostasecelular e do metabolismo energético.

A estabilidade conformacional das proteínas, ou seja, a manutenção do seu estado nativo é dependente do ambiente em que evoluíram para funcionar. Assim, variações deste ambiente, através de mudanças em faixas estreitas de temperatura, concentração de solutos ou pH resultam em desestabilização do estado enovelado das proteínas e consequentemente perda da sua capacidade funcional. A iniciação e manutenção do correto dobramento proteico surgiram durante o desenvolvimento da vida na Terra, garantindo o sucesso da atividade celular frente aos desafios ambientais, químicos e físicos, possibilitando maquinarias celulares cada vez mais complexas (Kultz, 2005).

Estresses ambientais que ameaçam a integridade do proteoma de um organismo ativam a resposta a proteínas desdobradas (UPR, do inglês, *unfolded protein response*). A UPR tenta cessar o acúmulo de proteínas desdobradas inibindo a transcrição de proteínas no retículo endoplasmático (ER) e aumentando a remoção de proteínas mal dobradas pela degradação lisossomal (Shen*et al.*, 2001; Kozutsumi*et al.*, 1988). Além disso, a síntese de chaperonas é aumentada, para aliviar a carga proteica desdobrada, a partir da deflagração da resposta de choque térmico (HSR, do inglês, *heat shock response*) (Akerfelt; Morimoto; Sistonen, 2010). As chaperonas são proteínas que atuam: prevenindo a agregação de proteínas mal dobradas, no redobramento de proteínas desnaturadas, no dobramento de proteínas *de novo*, notransporte intracelular de proteínas e auxiliando na proteólise (Hartl; Bracher; Hayer-Hartl, 2011). Se a adaptação da célula através da UPR não for suficiente para lidar com o aumento da carga de proteínas desdobradas, ocorre

a ativação do braço inflamatório desta via, com aumento de transcrição da proteína Jun-c terminal-n quinase (JNK) e das proteínas caspases, guiando a célula a uma resposta pró-inflamatória e pró-poptótica (Uranoet *al.*, 2000). Embora a adaptação transitória ao estresse agudo, obviamente, seja uma vantagem imediata de sobrevivência, as respostas inflamatórias ativadas, a longo prazo, por um estresse crônico, podem se tornar um problema para o organismo.

A ativação da HSR está associada à liberação, à trimerização e à conseguinte translocação do fator de transcrição de proteínas do choque térmico-1 (HSF-1), que depende do aumento da atividade e expressão da via da sirtuína 1 (SIRT-1) (Singh; Haslay, 2013). O impacto principal da ativação do HSF-1 é a elevada produção das proteínas de choque térmico (HSP), cujo principal representante é a proteína da família de 70 kDa (HSP70) (Tangeet *al.*, 2013; Wuet *al.*, 2013). As HSPs são chaperonas moleculares e estão amplamente presentes entre os organismos, desde o último ancestral comum universal (LUCA) (Reubeaudet *al.*, 2021), e são primordiais não só para a manutenção da proteostase, pelo seu papel de chaperona, mas, também, para a resolução da resposta inflamatória.

A ocorrência de uma resposta inflamatória completa depende da indução de proteínas pela ativação dos fatores de transcrição nucleares da família kB (NF-kB) (Oeckinghaus; Ghosh, 2009), que conduzem o processo inflamatório durante sua fase inicial e, simultaneamente, promovem sua resolução. Um dos mecanismos envolvidos no término da inflamação é, justamente, o disparo da HSR. A HSR é crítica para promover a resolução da inflamação, impedindo que se torne crônica. No entanto, parece que a resposta de choque térmico está severamente comprometida em tecidos metabólicos, tais como, tecido adiposo, fígado, músculo - justamente durante a inflamação crônica (Newsholme; Homem de Bittencourt Jr, 2016). Situação totalmente prejudicial ao organismo, tendo em vista que a HSP70, que deveria ser expressa em resposta ao estresse metabólico do próprio tecido adiposo, é um bloqueador fisiológico das vias de sinalização a jusante das JNKs, proteínas pró-inflamatórias que contribuem para a inibição da sinalização da insulina (Miragem; Homem de Bittencourt Jr, 2017). Estudos com indivíduos submetidos à cirurgia bariátrica

mostram ainda que o processo inflamatório que se inicia no tecido adiposo propaga-se para o fígado levando a uma redução na expressão de HSF-1, HSP70 e aumento expressivo na atividade das JNKs, tanto no fígado, quando no tecido adiposo (Di Naso *et al.*, 2015), o que poderia contribuir com a perpetuação da inflamação sistêmica (Newsholme; Homem de Bittencourt Jr, 2014). Desta forma, a baixa atividade da via da HSR se relacionaria diretamente com a resistência à insulina (IR) e o agravamento do *diabetes mellitus* do tipo 2 (DMT2) (Calapre; Gray; Ziman, 2013; Chung*et al.*, 2008), sendo, assim, de suma importância o entendimento da evolução temporal do quadro.

Abordagens farmacológicas (e.g., com o derivado de hidroxilamina BGP-15), bem como fisiológicas (e.g., tratamento hipertérmico) poderiam ser utilizadas de forma terapêutica nas complicações relacionadas às obesidades e DMT2 (Miragem; Homem de Bittencourt Jr, 2017; Hooper, 1999; Krause *et al.*, 2015), especialmente para pessoas com obesidade severa (grau III) às quais o acesso ao exercício físico é inicialmente dificultado. O tratamento hipertérmico, por si só, tem demonstrado inúmeros efeitos benéficos para o organismo por gerar redução na deposição de gordura, da IR e massa corpórea total (Krause *et al.*, 2015). A primeira indicação de uso terapêutico do choque térmico foi relacionada à sua capacidade de reduzir a glicemia de humanos diabéticos submetidos a sessões de sauna (Hooper, 1999). Evidências sugerem o potencial da terapia hipertérmica como uma modalidade de tratamento para doenças crônico-degenerativas relacionadas à obesidade (Brodmerkel; Taylor, 2016).

É importante ressaltar que os mecanismos fisiológicos relacionados ao controle do metabolismo energético foram selecionados visando respostas frente à escassez nutritiva e não ao excesso como ocorre na atualidade. Doenças metabólicas e inflamatórias crônicas, como a obesidade e suas comorbidades, ocorrem em decorrência deste desbalanço nutricional e têm tido uma incidência cada vez maior. Dados do ministério da saúde brasileiro indicam que 36 % dos adultos apresentam sobrepeso e mais de 21 % das pessoas sejam obesas (Ministério da Saúde, 2022).Obesidade, resistência à insulina e DMT2 estão fortemente associados à inflamação crônica

caracterizada pela produção anormal de citocinas pelo tecido adiposo (TA) visceral (Homem de Bittencourt Jr; Newsholme, 2015; Newsholme; Homem de Bittencourt Jr, 2014).

A resposta inflamatória que surge durante o desenvolvimento da obesidade, provocada pelo acúmulo de lipídeos e excessiva aporte de outros nutrientes ao TA, espalha-se para tecidos musculares, fígado e vasos sanguíneos (Miragem; Homem de Bittencourt Jr, 2017). Essa sobrecarga de nutrientes no TA ativa a UPR, causando a contínua ativação do NF-κB e do perfil inflamatório secretor de citocinas conhecido como: fenótipo de secreção associado à senescência (SASP do inglês, senescence-associated secretory phenotype) (Biden*et al.*, 2014). Pois, como os estímulos inflamatórios não cessam e levam ao estado de senescência proliferativa, a partir da ativação do inflamassomo NLRP3 (do inglês, NOD-like receptor pyrindomain-containing protein 3), uma alternativa à morte celular por apoptose (Cnop; Foufelle; Velloso, 2012). A ativação desse inflamassomo parece levar à destruição da proteína do antígeno humano R ligante de mRNA (HuR), responsável para a estabilização do RNA mensageiro do HSF-1, portanto, bloqueando a HSR no tecido (Newsholme; Homem de Bittencourt Jr, 2014). Assim, a obesidade conduz à ativação de vias de sinalização inflamatórias, contribuindopara o desenvolvimento e manutenção da inflamação crônica de baixo grau (Newsholme; Homem de Bittencourt Jr, 2014; Rius *et al.*, 2012). E, conforme comentado anteriormente, a HSR parece ser central para a resolução desse quadro.

HIPÓTESES

A hipótese dessa tese é que a resposta ao choque térmico está correlacionada ao nível de sensibilidade à insulina. E a HSR poderia ser medida de maneira representativa a partir do desafio térmico das células imunológicas do sangue. Além disso, terapia hipertérmica deve ser capaz de melhorar os índices de composição corporal e glicêmicos alterados pela indução da obesidade por dieta hiperlipídica.

OBJETIVOS

Artigo 1

Objetivo Geral

Sintetizar os dados da literatura sobre o histórico do estudo da resposta de choque térmico na proteostase e metabolismo energético por meio de uma revisão narrativa.

Objetivos Específicos

Descrever as vias envolvidas no controle da *caloristase;*

Revisar fatores e vias que influenciam no disparo da resposta ao choque térmico;

Relacionar evolutivamente mecanismos das chaperonas e do controle energético.

Artigo 2

Objetivo Geral

Sumarizar os achados publicados sobre resposta de choque térmico na resolução da inflamação e no desenvolvimento de doenças crônico-inflamatórias a partir de uma revisão narrativa.

Objetivos Específicos

Discorrer sobre a relação da resposta de choque térmico com a homeostase do organismo;

Descrever a relação da HSR na resolução da inflamação e sua supressão em doenças inflamatórias.

Artigo 3

Objetivo Geral

Sumarizar produções e propostas relacionadas aos seus métodos de indução e aferição da resposta de choque térmico em uma revisão narrativa.

Objetivos Específicos

Compilar propostas de estratégias físicas, farmacológicas e nutricionais para melhora da resposta de choque térmico;

Relatar a associação entre microbiota e integridade da HSR.

Artigo 4

Objetivo Geral

Acompanharo desenvolvimento da obesidade, o perfil de sensibilidade à insulina relacionada à capacidade de resposta ao choque térmico.

Objetivos Específicos

Avaliar a HSR por desafio térmico nas células do sangue total em camundongos C57BL/6J no desmame, tratados com dietas padrão ou hiperlipídica por 1, 4, 8, 10, 14, 18 ou 22 semanas.

Artigo 5

Objetivo Geral

Investigar os efeitos da intervenção com tratamento térmico em animais obesos resistentes à insulina.

Objetivos Específicos

Avaliar a HSR por desafio térmico nas células do sangue total em camundongos C57BL/6J mantidos por 18 e 22 semanas com dieta hiperlipídica ou padrão e submetidos a tratamento hipertérmico ou não.

Capítulo I

Título: The dance of proteostasis and metabolism: Unveiling the caloristatic controlling switch

Periódico: Cell Stress and Chaperones

Situação: Publicado em fevereiro de 2024.

(disponível em: <https://doi.org/10.1016/j.cstres.2024.02.002>)

Cell Stress and Chaperones (2024) 29:175–200
https://doi.org/10.1016/j.cstres.2024.02.002

REVIEW

The dance of proteostasis and metabolism: Unveiling the caloristatic controlling switch

Helena Trevisan Schroeder[1,a] · Carlos Henrique De Lemos Muller[2,b] · Thiago Gomes Heck[3,c] · Mauricio Krause[2,d] · Paulo Ivo Homem de Bittencourt Jr[1,*,e]

Received: 15 September 2023 / Revised: 2 February 2024 / Accepted: 4 February 2024

Abstract

The heat shock response (HSR) is an ancient and evolutionarily conserved mechanism designed to restore cellular homeostasis following proteotoxic challenges. However, it has become increasingly evident that disruptions in energy metabolism also trigger the HSR. This interplay between proteostasis and energy regulation is rooted in the fundamental need for ATP to fuel protein synthesis and repair, making the HSR an essential component of cellular energy management. Recent findings suggest that the origins of proteostasis-defending systems can be traced back over 3.6 billion years, aligning with the emergence of sugar kinases that optimized glycolysis around 3.594 billion years ago. This evolutionary connection is underscored by the spatial similarities between the nucleotide-binding domain of HSP70, the key player in protein chaperone machinery, and hexokinases. The HSR serves as a hub that integrates energy metabolism and resolution of inflammation, further highlighting its role in maintaining cellular homeostasis. Notably, 5′-adenosine monophosphate-activated protein kinase emerges as a central regulator, promoting the HSR during predominantly proteotoxic stress while suppressing it in response to predominantly metabolic stress. The complex relationship between 5′-adenosine monophosphate-activated protein kinase and the HSR is finely tuned, with paradoxical effects observed under different stress conditions. This delicate equilibrium, known as caloristasis, ensures that cellular homeostasis is maintained despite shifting environmental and intracellular conditions. Understanding the caloristatic controlling switch at the heart of this interplay is crucial. It offers insights into

Abbreviations: AMPK, 5′-adenosine monophosphate-activated protein kinase; BAG, *B*cl-2-*a*ssociated athanogene antiapoptotic nucleotide exchange factor; CHIP, C-terminal HSP70 binding protein; DNP, 2,4-dinitrophenol; ER, endoplasmic reticulum; GSK-3β, glycogen synthase kinase-3β; HBOT, hyperbaric oxygen therapy; HBP, hexosamine biochemical pathway; Hop, HSPC/HSP90-organizing protein; HSE, heat shock element; HSF1, heat shock transcription factor 1; HSP, heat shock protein; HSP70, the 70 kDa family of heat shock proteins; HSR, heat shock response; HuR, human antigen R, a.k.a. ELAV-1, for *e*mbryonic *l*ethal, abnormal *v*ision, Drosophila, homolog-like protein-1; IκB, inhibitors of κB transcription factors; JNK, c-Jun *N*-terminal kinase; NEF, nucleotide exchange factor; NF-κB, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; NLRP3, NOD-like receptor pyrin domain-containing protein-3 inflammasome; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; SIRT1, NAD^+-dependent deacetylase sirtuin-1; SNS, sympathetic nervous system; UPR, unfolded protein response

* Paulo Ivo Homem de Bittencourt Jr
pauloivo@ufrgs.br

[1] Laboratory of Cellular Physiology (FisCel) Department of Physiology, Institute of Basic Health Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil

[2] Laboratory of Inflammation, Metabolism and Exercise Research (LAPIMEX), Department of Physiology, ICBS, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil

[3] Post Graduate Program in Integral Health Care (PPGAIS-UNIJUÍ/UNICRUZ/URI), Regional University of Northwestern Rio Grande Do Sul State (UNIJUI) and Post Graduate Program in Mathematical and Computational Modeling (PPGMMC), UNIJUI, Ijuí, Rio Grande do Sul, Brazil.

[a] ORCID ID: 0000-0003-4000-3671
[b] ORCID ID: 0000-0003-0205-7775
[c] ORCID ID: 0000-0002-1242-5423
[d] ORCID ID: 0000-0001-9814-742X
[e] ORCID ID: 0000-0003-1907-3341

Published online: 6 February 2024

a wide range of conditions, including glycemic control, obesity, type 2 diabetes, cardiovascular and neurodegenerative diseases, reproductive abnormalities, and the optimization of exercise routines. These findings highlight the profound interconnectedness of proteostasis and energy metabolism in cellular function and adaptation.

Keywords Heat shock response · Proteostasis · Energy metabolism · 5′-AMP-activated protein kinase · Caloristasis · Cellular homeostasis

Introduction

The heat shock response (HSR) is a highly evolutionarily conserved cellular manifestation primarily devoted to re-establishing cellular homeostasis after stressful situations. Although the HSR acts as a frontline defense against factors that can denature proteins, that is, proteotoxic challenges (e.g., heat, heavy metals), it was evident since the pioneering studies of Professor Ferruccio Ritossa[1–3] that this response is also triggered by disruptions in energy metabolism, such as oxygen deprivation and uncoupling of ATP synthesis. This insightful belief of Ritossa becomes a cogent line of reasoning that gains clarity when considering the following factors: protein synthesis demands significant energy expenditure; rectifying misfolded proteins via the HSR requires ATP energy; expediting mitochondrial ATP production amplifies the generation of proteotoxic reactive oxygen species (ROS). Hence, it is now more than expected that the HSR had evolved alongside the regulatory principles of cellular energy metabolism.

Evidence suggests that the emergence of proteostasis-defending systems dates back to last universal common ancestor (LUCA), more than 3.6 billion years ago,[4] exactly at the same time that sugar kinases started evolution to improve glycolytic efficiency, approximately 3.594 billion years ago.[5] Therefore, it is not surprising that the evolution of the nucleotide-binding domain (NBD) of the 70 kDa heat shock protein (HSP70, the working force of protein chaperone machinery) had introduced subdomains (Ia and IIa, discussed below) that are spatially identical to the NBD of hexokinases[6] and other sugar kinases.[7]

It has been known for a long time that the HSR acts as a hub to integrate *energy metabolism* and the *resolution of inflammation* because the HSR pathway is anti-inflammatory *per se*.[8] For instance, 5′-adenosine monophosphate-activated protein kinase (AMPK), which is the master cellular energy sensor activated during metabolic stress (energy paucity), simultaneously assumes the role of a central regulator of anti-inflammatory responses through the inhibition by phosphorylation of glycogen synthase kinase-3β (GSK-3β). This phosphorylation event liberates the heat shock transcription factor 1 (HSF1), initiating the HSR because GSK-3β constitutively inhibits HSF1.[8–10] Conversely, the NAD^+-dependent protein deacetylase of class III family sirtuin-1 (SIRT1), which is activated by calorie restriction, enhances the HSR by increasing HSF1 protein expression and DNA-binding activity onto heat shock genes.[11] Actually, heat shock and calorie restriction act synergistically to arm an HSR.[12] In addition to enhancing the expression of protein chaperones, HSF1 directly increases the expression of an ample array of genes implicated in energy metabolism, such as the SIRT1 itself, AMPK, and peroxisome proliferator-activated receptor-γ coactivator-1α, just to mention a few.[13,14] Apart from the expression of glucose-regulated chaperones (e.g., GRP78 and GRP75), the HSR is also connected to energy metabolism through the hexosamine biochemical pathway (HBP, discussed below), a metabolic shunt from glycolysis that leads to the blockade of GSK-3β activity and enhanced DNA-binding activity of HSF1.[15]

While the role of AMPK in activating the HSR during *predominantly proteotoxic* stress is well-established, a growing body of evidence is challenging this notion by highlighting AMPK's potential to exert opposing effects. A case in point is the involvement of the mRNA-binding protein HuR, also known as embryonic lethal abnormal vision Drosophila homolog-like protein-1. HuR plays a pivotal role in stabilizing SIRT1 mRNA, consequently promoting the expression and activity of HSF1, a key player in the HSR. To effectively carry out its functions on target transcripts, HuR must translocate from the nucleus to the cytoplasm. Herein lies an intriguing paradox: contrary to its expected role, AMPK, which is typically known to promote SIRT1 activation during an HSR-dependent anti-inflammatory response,[8] actually hinders the nuclear export of HuR,[16–18] thereby attenuating the vigor of the HSR. Reinforcing this line of thought, Dai and colleagues[19,20] have provided compelling evidence indicating that AMPK, when confronted with *predominantly metabolic* stress, actively suppresses the proteostasis-preserving facet of the HSR. In contrast, during a bona fide HSR event (predominantly proteotoxic), the orchestrated activation of protein phosphatase 2A by HSF1 leads to the inhibition of AMPK,[21] subsequently fostering an elevation in HSP70 expression.[22]

These findings underscore a nuanced equilibrium governing the orchestration of the proteotoxic stress response and the metabolic stress response, operating at

the interface of AMPK and HSF1 to regulate the HSR under diverse stress conditions. This intricate modulation of cellular stress responses was meticulously explored by Swan and Sistonen[23] based on the findings of Dai's group[19] and now is referred to as *caloristasis*.[24] In fact, predominantly *proteotoxic stress* and predominantly *metabolic stress* work in opposition to maintaining a very delicate thermodynamical poise that warrants the steady state between energy homeostasis and proteostasis so that overall cellular homeostasis may take place whatever the environmental or intracellular conditions. The central caloristatic controlling switch that integrates proteostatic and metabolic stressful conditions is summarized in Figure 1.

Taking into account the above findings, the purpose of the present work is to illustrate the currently recognized interconnections between the HSR as a safeguarding mechanism for proteostasis and the pathways that govern energy metabolism. As we shall discuss herein, understanding the caloristatic controlling switch is a fundamental prerequisite that may enable us to delve into the intricacies underlying a wide array of conditions, ranging from glycemic control, obesity, type 2 diabetes mellitus, cardiovascular and neurodegenerative diseases, to human reproduction abnormalities and well-structured exercise routines.

Unraveling the mosaic: convergence of insights leading to contemporary understanding of the HSR

In order to understand the logic of the HSR, it is interesting to note the history of heat shock proteins (HSPs) that can be traced back to the observations of

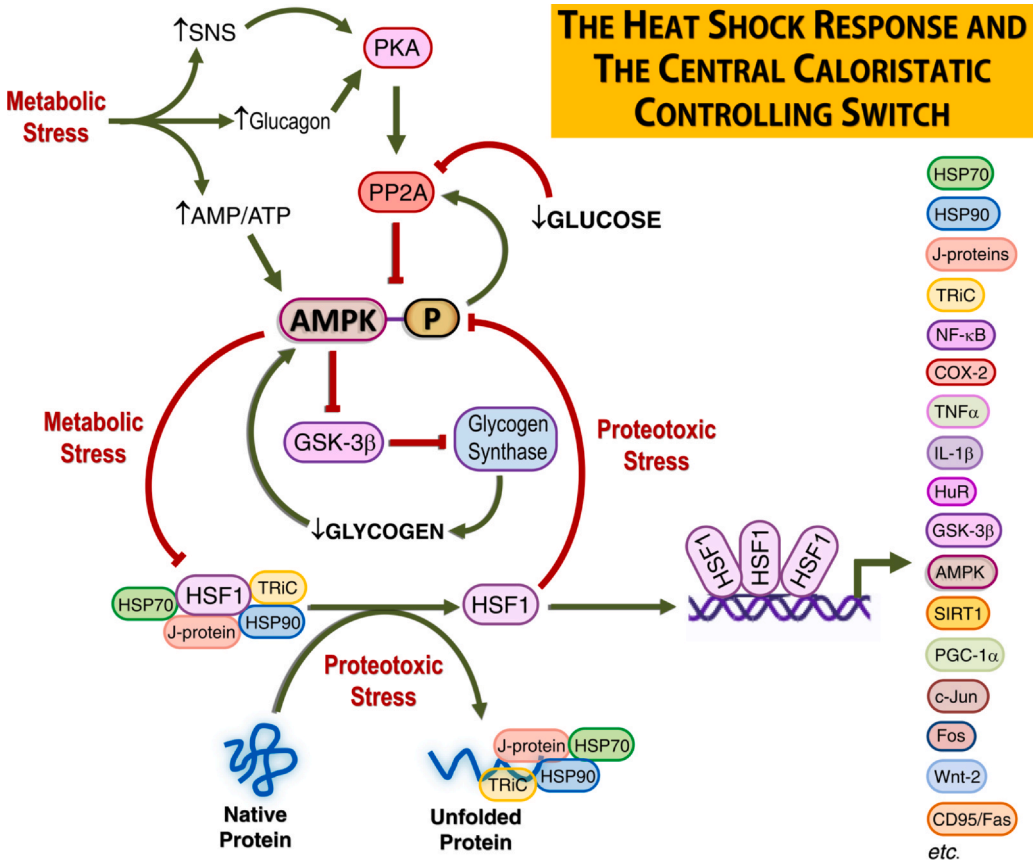


Fig. 1 The heat shock response and the central caloristatic controlling switch. This switch orchestrates a delicate dance between metabolic stress, characterized by the activation of AMP-activated protein kinase (AMPK), and proteotoxic stress, marked by the accumulation of misfolded proteins and oxidative pressure. During periods of predominantly metabolic stress, AMPK takes center stage. It exerts its influence by phosphorylating heat shock transcription factor 1 (HSF1) at Ser121, effectively hindering the transcription of HSF1-dependent genes. On the other hand, predominantly proteotoxic stress ushers in a different set of players. The accumulation of misfolded proteins and heightened oxidative stress directly activate HSF1. Consequently, under proteotoxic stress conditions, a substantial portion of HSF1 is liberated from chaperone machines, effectively blocking AMPK and allowing for the expression of over 5200 genes directly regulated by HSF1. AMPK, 5′-adenosine monophosphate-activated protein kinase; GSK-3β, glycogen synthase kinase-3β; HSP70, the 70 kDa family of heat shock proteins; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; SNS, sympathetic nervous system.

Ferruccio Ritossa, a young geneticist at the time, which laid the foundation for our comprehension of these important proteins. He noticed that polytene chromosomes of salivary glands of *Drosophila busckii* fruit-fly larvae exhibited a novel pattern of puffing when heat-shocked from normal (25 °C) to 30 °C (or higher) in an incubator.[1] It is worth noting that polytene chromosomes are formed through the successive duplication of each chromosomal element (chromatid) without their separation from the chromosomes of diploid nuclei.[25] Consequently, the newly formed chromatids remain connected lengthwise and collectively give rise to cable-like structures known as polytene chromosomes. Importantly, back in 1962, the study of gene expression was limited to systems such as *Drosophila spp*. polytene chromosomes, where gene activity was visible as chromosomal puffing under the light microscope.[26] Messenger RNA had only just been discovered by Professor Matthew Meselson's laboratory in 1961.[27]

Ritossa supposed that the rapid appearance of specific puffs and disappearance of others in 3–4 min after the inadvertent but serendipitous heat shock of *Drosophila* cells and tissues would be related to the synthesis of mRNA since tritiated cytidine incorporations into the puffs were abolished by RNAse treatment.[1] He also realized that the effect of heat shock, leading to the appearance of some puffs and reversal of others that were present under normal conditions, was mimicked by the treatment of cells, organs, or whole organisms of *Drosophila spp*. with uncouplers of ATP synthesis, such as 2,4-dinitrophenol and sodium salicylate.[1–3] Moreover, although some effects could be observed when larvae grown at 19 °C were exposed to 25 °C, they were typically of low intensity and efficiency. This suggested that variations in puffing patterns might not be solely dependent on a temperature increase of 5–6 °C, but rather on the rapid attainment of a specific temperature threshold. Therefore, Ritossa conjectured that these responses should be of general importance, especially because he had the understanding of *Drosophila spp*. as "*somehow between bacteria and man*" and also because similar results were obtained with anaerobiosis, thus linking the observed *heat shock response* to *energy production*.[26]

At that time, Michael Ashburner, who had been studying the puffing patterns of *Drosophila spp*. since the late 1950s at the University of Cambridge (UK), was investigating the induction of specific puffs during the stages of development of the animal[28,29] as well as by temperature shock, hormonal treatment, and anaerobiosis.[30] He had some suspicion about the likely expression of particular proteins related to these puffs due to the following observations. Incubation of salivary glands *in vitro* with antibiotic inhibitors of protein synthesis (such as cycloheximide and puromycin) did not affect puffing in *D. melanogaster*, but injecting cycloheximide or puromycin into 90-h L3 larvae had significant inhibitory effects on puffing. Also, these treatments rapidly inhibited the incorporation of amino acids into proteins (in less than 1 min), although changes in puffing patterns took approximately 3 h to become apparent.[30] Then, Alfred Tissières, on a sabbatical visit to the laboratory of Ursula M. Tracy at the California Institute of Technology, confirmed the appearance of new bands separated by SDS-PAGE in ^{35}S-methionine-labled tissues of *Drosophila melanogaster*; the same was observed when the whole animal was injected with the radiotracer.[31] By using ^{3}H-uridine labeling, they were also able to associate the synthesis of new polypeptides with the puffing activity of polytene chromosomes. They were impressed by the remarkably high rate of protein synthesis observed in a single band, later identified as ~70 kDa.[32] This one band alone accounted for approximately 15% of all the labeled bands induced by heat shock. In addition, as previously noticed by Ritossa, heat shock induced the appearance of new proteins and the disappearance of others.[31]

The Ashburner group working on *D. melanogaster*, *D. hydei*, and *D. simulans* confirmed the above observations and added new information to them, showing that actinomycin D (a transcription inhibitor that binds to DNA duplexes preventing RNA polymerase elongation) blocks the synthesis of heat-induced proteins *only* if added at the beginning of the shock.[32] Furthermore, in this latter study, Ritossa's former notion that *energy-threatening situations* and *heat shock* should mandatorily share some fundamental principle in common was also corroborated. Accordingly, Lewis and co-workers[32] tested the effects of uncouplers of oxidative phosphorylation (2,4-dinitrophenol), inhibitors of electron transport (rotenone), or recovery from prolonged anaerobiosis (2 h under nitrogen atmosphere) and, in fact, observed exactly the same patterns of puffing paralleled by the synthesis of new proteins, in particular, one of 70–72 kDa, which was always present in such preparations.

Susan Lindquist (then Susan Lee Lindquist McKenzie) discovered at Meselson's laboratory that *Drosophila* cells exhibited the same response to heat shock as reported by Tissières and colleagues in 1974,[31] with translational and transcriptional mechanisms governing the response.[33–36] These findings were and still are considered the most robust and widespread change in eukaryotic gene expression so far. She also revealed the unexpected capacity of eukaryotic cells to discriminate between coexisting mRNAs and independently regulate their translation. In other words,

during heat shock, cells efficiently translate heat shock mRNAs while blocking normal mRNAs from translation, yet holding them ready for reactivation after heat shock. Lindquist's observations then explained the findings of Ritossa and Tissières' group that heat shock induces an extraordinary appearance of some puffs and the vanishing of others in polytene chromosomes, now under the molecular biology point of view.

Notwithstanding, Susan Lindquist was, to the best of our knowledge, the first to employ the term "*heat shock proteins*" to this set of heat-induced polypeptides and to emphasize the importance of the 70,000-dalton HSPs.[33] Since then, many groups have exploited details on the expression of heat shock proteins or heat shock-induced peptides, including Tissières' laboratory[37] and Ashburner's group, the latter introducing the acronym *HSP* to differentiate such polypeptides: HSP70, HSP80, and so on.[38]

At that time, there was a primary source of confusion surrounding HSPs with molecular weights around 70 kDa. Mirault and colleagues[37] conducted two-dimensional gel experiments that revealed multiple polypeptides in this range, spanning from 70 to 72 kDa. Genetic and biochemical evidence indicated that these were very similar proteins. Therefore, two possible explanations were postulated: post-translational modification (PTM) of a parent polypeptide or each protein being the product of a particular gene copy due to multiple copies of the coding sequences.[38] The latter has been proven to be the case, as approached below.

Lindquist's research group made significant contributions to the understanding of the role of HSPs in preventing and repairing stress-induced protein damage and its consequent cell toxicity. Her findings demonstrated that HSPs play a critical role in turning off the mechanisms that are activated in response to stress at every level. As HSPs restore protein homeostasis (proteostasis) and reset the damaged regulatory systems that inhibit normal mRNA functions, the elegant logic of the regulatory circuitry is revealed. By restoring regulatory systems to their normal state, HSPs eliminate their own advantage and switch off the response.[39] Lindquist's work, in conjunction with the seminal work of Spradling, Pelham, Lis, and Wu on transcription, has resulted in the HSR being regarded as one of the most beautiful and complete examples of eukaryotic gene regulation.[39]

More recently, there have been notable observations regarding the HSR. The universality of the response and the conservation of inducible genes across different species throughout evolution have been recognized as significant findings. These observations indicate that HSPs play fundamental roles in biological processes,[40] as previously assumed by Ritossa in his initial works[1–3] that were not widely accepted at that time, by the way.[26] Indeed, the HSR has proved to be a universal phenomenon observed in every organism where it has been sought, ranging incredibly from eubacteria to archaebacteria, and from mice to soybeans.[41] Moreover, it has been known for a long time that the HSR is influenced by the metabolic state of the cells. For instance, in the yeast *Saccharomyces cerevisiae*, the HSR in fermenting cells grown at 25 °C is transient at 36 °C and sustained at 40 °C, while in respiring cells, the response is transient at 34 °C and sustained at 36 °C. These findings resemble in much the effects of hyperbaric oxygen therapy observed in mammalian cells,[24] as we shall approach later. Additionally, the synthesis of HSPs is determined by previous incubation temperatures: in *Drosophila* cells, when the temperature is abruptly increased to high levels, the maximum response occurs at 37 °C, and only minimal synthesis is observed at 39 °C. However, when the temperature is gradually increased (a regimen more likely to reflect natural environmental exposure), the response is extended over several degrees. Apart from a few exceptionally rare and evolutionarily justifiable cases (discussed next), these discoveries have been replicated in several other organisms.[41]

These proteins are widely regarded as among the most highly conserved proteins in existence.[42] Under normal growth temperatures, the HSP70 gene is transcribed at low levels in human cells. However, a transient heat shock at 43 °C can induce the gene by over 20-fold.[43] Importantly, the unusually high degree of conservation observed in the amino acid sequence of human HSP70,[43] which was found to be *73%* identical to that of the *Drosophila* HSP70 and *47%* identical to the *Escherichia coli* dnaK (!), suggests that HSP70s were conserved throughout evolution for a specific and important reason over the last 3.6 billion years.

Apart from certain stenothermal organisms, which are only capable of surviving within a narrow range of ambient temperatures (such as those found in the deep sea and polar environments), all eurythermal organisms (including birds and mammals) that have been studied so far exhibit the *inducible* production of proteins encoded by the HSP70 and HSP90 gene families in *response to elevated temperatures*. Interestingly, the stenothermal Antarctic fish Emerald rockcod (*Trematomus bernacchii*) possesses an exceptionally unusual trait: the animal *does not* express inducible forms of HSPs when subjected to heat or heavy metal stress. Instead, when challenged with non-lethal thermal stress, this fish increases the synthesis of the constitutive HSP70. This unique trait is exclusively found in animals that inhabit subzero, thermally stable waters.[44] It is believed that these animals *lost* the HSR between 14 and 25 million years ago. This

timeframe coincides with the opening of the Drake Passage, leading to a significant cooling of Antarctic waters.[44] As a result, this evolutionary adaptation occurred relatively recently in terms of both evolution and geological history. A similar phenomenon is observed in the freshwater coelenterate species *Hydra attenuata* when it faces thermal or heavy metal stress. Notably, only the constitutively expressed HSP60 (but not HSP70), which functions as the primary chaperone, experiences an enhanced synthesis in such conditions. This heightened synthesis of HSP60 coincides with an increased thermotolerance, allowing the organism to better withstand subsequent challenges. Quirkier, its congener species *Hydra oligactis* displays an extreme sensitivity to thermal stress and fails to express any HSP in response to heat at all.[45] Indeed, many animals, particularly the ectothermic ones, evolved resourceful strategies to survive adverse ambient conditions, and all of them employ changes in chaperone functions.[46]

Shortly after cloning, it became evident that the human HSP70 gene belongs to a multigene family, similar to the 70 kDa multigene families found in *Drosophila* and yeast,[47] reaffirming one of the hypotheses raised by Ashburner's laboratory.[38] Notably, *HSPA1A*, the gene responsible for coding HSP70, is an intronless gene. This lack of intervening sequences is not just a coincidence, but a significant characteristic for genes whose transcripts accumulate (or must accumulate) rapidly in the cytoplasm. In contrast, most eukaryotic genes containing intervening sequences require proper processing before their transcripts can be transported into the cytoplasm. This is not the case for *hspa1a*, and it explains the rapid transcription and accumulation rates observed in *Drosophila* HSP70 mRNAs.[47]

Before delving further into this discussion, it is pertinent to highlight that the term "*proteotoxicity*" was initially coined by analogy to "genotoxicity." It was first introduced by Professor Larry Hightower[48] to characterize the harm inflicted upon proteins by various chemical and physical agents.

Initially identified as stress-responsive proteins necessary to cope with thermal and other proteotoxic stresses, the human family of HSPs was soon found to contain members that are constitutively expressed, such as HSC70, the cognate form of HSP70, also known as HSP73 encoded by *HSPA8* gene, besides its most prominent stress-inducible HSP72, encoded by *HSPA1A* and *HSPA1B* genes.[49] Another feature of HSPs is their location in different compartments of the eukaryotic cell. In addition, they may or may not be induced by heat and this depends on tissue and *metabolic state*. For instance, glucose-regulated protein 78 (GRP78), a member of the HSP70 family, is situated within the endoplasmic reticulum (ER). Its expression is induced in response to diminished glucose levels. In contrast, another glucose-responsive HSP70, Glucose-Regulated Protein 75 (GRP75), resides in the mitochondrion. GRP75 similarly reacts to glucose scarcity but remains unresponsive to heat shock.[41,42]

Insights into the true function of HSPs as proteostasis-safeguarding in the cells, nevertheless, emerged with the discovery that abnormal, denatured proteins can act also as stress signals, activating heat shock genes.[50] Inside cells, HSPs play a vital role in preventing protein aggregation when newly synthesized proteins are leaving the ER *en route* to their final destination, or when intracellular proteins are under immediate threat of misfolding due to various agents such as heat, alcohol, amino acid analogs or heavy metals, and post-ischemic reperfusion, which induce oxidative stress and menace protein physiology as well. However, it was difficult to solve the puzzle that connected the spatial conformation of polypeptides with the activation of specific genes. Even more: how did evolution prepare sensors, if any, capable of transducing the misfolding of a protein into a signal for the transcription of heat shock genes?

For a long time, it was believed that the likelihood of a stepwise random folding-denaturation-refolding process during the biosynthesis of a polypeptide chain from the amino to carboxy-terminus would be minimal. Moreover, it was widely recognized that there exists a delicate balance between a stable, native protein structure, and a random, biologically functionless one. Therefore, it was expected that some biological system would operate to maintain this balance thermodynamically.[51] The question is that, in Anfinsen's *in vitro* experiments with chemical denaturants, the probability of a polypeptide folding correctly after the denaturant is removed increases when the protein concentration is low, limiting inter-polypeptide interactions, and at low temperatures, which attenuates hydrophobic interactions. However, this is not the case for real cells *in vivo*. The high protein concentrations and temperatures present within the cell can cause premature interactions between newly synthesized polypeptides, resulting in misfolding and aggregation.[52] This explains why, during the course of metazoan evolution, molecular chaperones emerged as a vital component in maintaining proper protein folding and preventing aggregation.[4]

The discovery of HSP chaperone cascades (next sections), nevertheless, does not alter Anfinsen's thermodynamic folding principle,[51] which states that the primary amino acid sequence encodes all the information necessary to determine the shape of the native state, and chaperones are not responsible for changing it.[53] Chaperones function in a manner similar to enzymes in that

they do not interfere with the quantum of free energy (ΔG) involved in folding. Instead, they lower the activation energy required for proper folding or transport toward other cellular compartments. This is made possible because, like conventional enzymes, chaperones "*embrace*" specific segments of the polypeptide chain, reducing their mobility (entropy). As expected, this energetic movement against spontaneity ($\Delta S < 0$) requires energy input obtained from ATP hydrolysis, but this is the faster step of the folding reaction. Instead, upon being released at their final destinations, Brownian mobility restores entropy (and free energy) to the medium, and this is the slow (bottleneck) step of the entire process.

For example, HSP70-HSP90 cascades significantly increase folding yields, but they basically do not affect the overall kinetics of the folding process.[54] This is because HSP70s dose-dependently block protein folding by imprisoning client proteins at the chaperone core, a very fast process that is ATP-dependent. HSP90s, on the other hand, promptly resume the folding course by taking over client proteins from HSP70s in an ATP-dependent manner as well. HSP70-HSP90 activities are limited to the early folding phase, during which their ATPase activity is required for a few seconds (k_{cat} of up to $0.79\ s^{-1}$).[55] Once this phase is complete, the client protein follows an Anfinsen folding trajectory (rate limiting for the velocity of folding; several minutes) that does not require the assistance of either chaperone.[53]

For the inter-organelle transport of polypeptides or dispatch of them from the ribosomal exit tunnel, a pulling force (ΔG) is required, which is generated by the low intrinsic entropy state created by the limited freedom of movement of polypeptides bound to HSP70 near the membrane and the pore (translocon) through which the polypeptides must pass. As the polypeptide moves inward through the translocon, it increases the freedom of movement ($\Delta S > 0$) of the substrate-bound HSP70, leading to an increase in entropy (resulting in a negative ΔG). This creates a one-way pulling motion, as entropy can only spontaneously increase.[56]

The available data suggest a sequential occurrence of ATP and cochaperone-induced structural rearrangements in bacterial HSP70 (DnaK), leading to the resolution of previously unforeseen cochaperone and client-induced changes.[57] Peptides induce significant conformational alterations in DnaK·ATP prior to ATP hydrolysis, whereas protein clients induce smaller changes but exhibit greater effectiveness in stimulating ATP hydrolysis.[57] The analysis of the enthalpies of activation for the ATP-induced opening of the DnaK lid in the presence of client peptides indicates that the lid *does not* exert an enthalpic pulling force on bound clients. This finding suggests that entropic pulling serves as a major mechanism for client unfolding,[57] as previously proposed.[53,54] These recent findings provide valuable insights into the mechanics, allostery, and dynamics of HSP70 chaperones, as demonstrated.[57] In essence, chaperoning is a process fundamentally governed by thermodynamics, much like the entire process of triggering the HSR.

While the HSR also extends to the organism level,[58] HSPs play two distinct roles in terms of cellular protein protection[56]: *housekeeping* and *stress-activated* functions. The housekeeping functions are related to (1) cooperation with other protein folding and quality-control machineries; (2) *de novo* protein folding; (3) protein translocation across membranes; (4) assembly/disassembly of protein complexes; (5) regulation of protein activity; and (6) protection from proteolysis. On the other hand, stress functions evolved to limit the noxious effects of unfolded proteins, which evoke inflammatory responses if accumulated.[11] They comprise (1) prevention of protein aggregation; (2) protein disaggregation, in cooperation with small HSPs (sHSPs) and HSP100 families; (3) protein refolding; and (4) protein degradation (e.g., autophagy and ubiquitin-proteasome system) to clear aberrant proteins and protein aggregates. Structure-function relations and HSP70-client protein interactions have also been detailed.[59] Finally, HSR regulatory mechanisms poise proteostasis protection with all metabolic pathways related to energy preservation (caloristasis), including animal reproduction and anti-inflammatory responses. This is why modern human diseases such as obesity, in which a surplus of energy is detected within cells, so dramatically disarrange proteostasis and HSR-dependent anti-inflammatory pathways.[58] After all, the HSR evolved to juxtapose proteotoxic stress with metabolic stress, specifically in response to indications of insufficient cellular energy, not energy surplus.

Comparing the initial observations described in this section with what is known at present, it is amazing what a close look at the result of an error in the laboratory gave us. On the occasion of the 50th anniversary of the discovery of the HSR, Professor Maria Gabriella Santoro talked through the significance of the discovery. In her reflections, she highlighted the first interpretation of the HSR given by Ritossa himself, which is worth mentioning: "*It did not matter if this interpretation was true or false; it was a working link between imagination and reality, like love*." Ferruccio is not only a scientist and an artist; he is also a poet, she said.[60]

Heat shock protein families

Before exploring the roles of HSPs, it is worth noting that, although the term "molecular chaperones" be

commonly used in the field of HSPs, it was originally introduced by Laskey and colleagues[61] to describe the remarkable capacity of these proteins to prevent incorrect ionic interactions between histones and DNA.

To cope with both housekeeping and stress-induced functions, evolution ameliorated, from ancient prokaryotic ancestors, eight families of HSPs. They were initially characterized by their molecular weight (i.e., HSP72, HSP90, etc.) and are now grouped into gene families and superfamilies, more consistently with HUGO Gene Nomenclature Committee as follows[49]: *HSPA* (e.g., HSP70), *HSPB* (small HSPs), *HSPC* (e.g., HSP90), *HSPD* and *HSPE* (HSP60 and HSP10 chaperonins), *HSPH* (e.g., HSP110), *DNAJ* (J-proteins, formerly HSP40 cochaperones) alongside *CCT*, and other chaperonin-related genes.

To date, 13 different genes have been identified in the human genome that code for members of the HSPA superfamily: 11 for HSPB, five for HSPC, one for HSPD, one for HSPE, nine for the CCT chaperonin genes, and three others for chaperonin-like (one for MKKS and two for BBS).[49] Although the number of HSPA homologs/paralogs in the human genome is notable, it is the number of identified DNAJ genes in humans[62] that truly stands out, with 50 genes identified (4 DNAJA members, 13 DNAJB, and 33 DNAJC). This high number is an indication of the significant role that DNAJ genes play in the chaperone machinery.[62] DNAJ expression is found in all cellular compartments.[63]

J-proteins are distinguished by the presence of a conserved J-domain responsible for recruiting and activating HSPA ATPase activity.[49] This domain, which is named after its founding member, the *E. coli* DnaJ cochaperone, contains a highly conserved ~70 amino acid signature region. Of particular importance is a His-Pro-Asp tripeptide, which is located within a loop connecting the two main helices (helix II and helix III). The His-Pro-Asp tripeptide motif is critical for the J-domain's function in stimulating HSP70's ATPase activity.[64]

The HSPA/HSP70 superfamily, in particular, exhibits a high degree of diversity, which can be attributed to several factors, for instance, the various possible cellular locations, such as cytoplasm and nucleus (e.g., HSPA1A/HSP72 and HSPA1B/HSP70-2), ER (e.g., HSPA5/GRP78), and mitochondrion (e.g., HSPA9/GRP75). Despite the significant homology between the genes in this superfamily, *HSPA* members demonstrate a high degree of specialization, nonetheless. For example, the response to stress can vary significantly: *HSPA8*/HSC73 (expressed in both cytoplasm and nucleus)[63] is primarily a non-inducible housekeeping gene, whereas *HSPA6*/HSP70B′ exhibits strictly inducible expression, with minimal or no basal expression in most cells. Actually, there is a vast repertoire of combinations between intracellular location and inducibility among HSP members.[65] Studies have shown that proteasome inhibition is a potent activator of HSP70B′. In comparison to HSP72, which is the primary responder to increasing levels of proteotoxic stress among the *HSPA*/HSP70 family, *HSPA6*/HSP70B′ serves as a secondary responder. Interestingly, in cell models, *HSPA6*/HSP70B′ is induced by $ZnSO_4$ but not HSP72, indicating that *HSPA6*/HSP70B′ may have a stressor-specific primary role.[66]

The ER HSPA5 member, also referred to as binding immunoglobulin protein (BiP) or GRP78 (78 kDa glucose-regulated protein), is a master regulator of the unfolded protein response (UPR), reducing ER stress levels and apoptosis due to an enhancement of the cellular folding capacity, including against prion replication.[67] The mitochondrial HSPA9, also known as GRP75 (75 kDa glucose-regulated protein), is a heat shock cognate protein that plays a capital role in cell proliferation, stress response, and maintenance of the mitochondrion. It is essential in increasing ER-mitochondria contact during palmitate-induced apoptosis in pancreatic β-cells.[68] The expression of both GRP78 and GRP75 is also activated by lowering glucose levels within the cell. Actually, the level of HSP expression may determine if a cell can be rescued from a death stimulus or must undergo apoptosis.[69]

Chaperones also differ much in their preference for client proteins. For instance, while cytosolic HSPA/HSP70s have an inclination for binding leucine-enriched peptide motifs, which are abundant in aliphatic residues, the ER homolog BiP/HSPA5 has a preference for motifs with aromatic residues.[56] Apropos of HSPA gene family, its members possess high but different degrees of homology in respect of *HSPA1*/HSP72 (the most highly expressed one), namely: *HSPA1B*/HSP70-2, 99%; *HSPA5*/GRP78, 64%; *HSPA6*/HSP70B′, 85%; *HSPA8*/HSP73/HSC70, 86%; *HSPA9*/GRP75, 52%.

The triggering of HSP production during proteotoxic stress depends on the launching of the activation of heat shock transcription factors (HSFs), with HSF1 being the most studied due to its direct link with proteotoxic stress and the universality of expression across species. Under non-stressful situations, HSF1 resides in the cytoplasm in a monomeric conformation that has *no* DNA-binding or transcribing activity. To be fully activated, HSF1 first needs to trimerize and subsequently gain DNA-binding activity, nuclear accumulation, and extensive PTMs, including multiple serine-phosphorylations, acetylation/deacetylation, or even sumoylation and ubiquitinylation, whose prevalence depends on the physiological context.[70,71] Furthermore, as mentioned

above, a sophisticated caloristatic control system ultimately governs HSF1's involvement in the intricate process of switching between proteostasis-preserving and energy-conserving mechanisms. In any way, the main impact of HSF1 activation is the high throughput of HSPs, which represent 5–10% of the total protein in most cells and their intracellular content can increase about 20 times in response to stressors.[72]

The heat shock factors (HSFs)

Unlike invertebrates, which possess a single HSF, plants and vertebrates express multiple HSFs. Humans have six members in the HSF family, namely HSF1, HSF2, HSF4, HSF5, HSFX, and HSFY.[71] Each HSF has unique and overlapping functions, tissue-specific expression patterns, and undergoes various PTMs while interacting with multiple protein partners.[73] In eukaryotes, HSF1 is involved not only in HSP gene expression and stress resistance but also in the expression of genes with roles in cell maintenance and differentiation, as well as in developmental processes.[13,14,74]

Some genes whose expression is dependent on the binding of activated HSF1 include members of different HSP families, HSPA/HSP70 (HSPA1A, HSPA1B, HSPA1L, and HSPA8/HSC73), HSPC/HSP90 (HSPC1/HSP90AA1 and HSPC3/HSP90AB1), HSPH (HSPH1/HSP105 and HSPH3/HSPA4L), DNAJ/HSP40 (DNAJA1, DNAJA4, DNAJB1), chaperonins (HSPE1/HSP10 and HSPD1/HSP60), small HSPs (HSPB1 and HSPB8), BAG3, and many other stress-responsive but not necessarily HSP genes.[75]

In fact, HSF1 also directly regulates the expression and activity of key factors involved in cell differentiation and longevity, autophagy, mitochondrial and ribosomal biogenesis, immune responses, multidrug resistance, cancer progression, aging, and neurodegenerative diseases.[13,70,71,73,76] This feat is accomplished through the activation of HSF1, which orchestrates the transcription of a remarkable array of more than 5200 genes. This comprehensive list includes all core chaperones and their associated cochaperones, the mRNA-binding protein HuR, and the transcription factor peroxisome proliferator-activated receptor-γ coactivator-1α, both of which play pivotal roles in the HSR. Additionally, HSF1 regulates the expression of pro-inflammatory genes such as cyclo-oxygenase-2, IL-1β, TNFα, and the master regulator of inflammation, NF-κB. Notably, HSF1 also influences metabolic processes by modulating AMPK, sirtuin-1, and GSK-3β. Moreover, it exerts control over cell cycle and differentiation through the regulation of c-Jun, Fos, Wnt 2, CD95/Fas, and cyclin-dependent kinase inhibitor 2B, among numerous others, as depicted on the far-right side of Figure 1.

In entirety, HSF1 exerts control over nearly 25,000 genes, either directly or indirectly, through its collaboration with other transcription factors. This control mechanism entails both the activation of specific genes and the inhibition of others.[13,14]

Initially, HSF1 was identified as the primary regulator of the HSR. However, it is now known that HSF2 modulates HSF1-mediated expression of HSP genes by forming heterocomplexes. Upon exposure to heat shock, HSF1 and HSF2 accumulate in nuclear stress bodies and bind to satellite III repeats.[73] Of note, HSF1 stimulates the transcription of HSF2,[13,14] so that HSF1 and HSF2 present a cross-regulation. In summary, HSF1 regulates protein quality-control machinery and gene expression to support cell survival, and this is modulated by the cellular metabolic status as well.[23,24] On the other hand, HSF2 is highly expressed during early development and in the testis, while it has multiple roles as an activator of protein chaperone genes, and as a tumor suppressor. HSF2 works as an activator of protein chaperone genes when the temperature is in the febrile range. HSF4 is required for eye lens development and is also expressed in the heart, brain, skeletal muscle, and pancreas. Recent research has revealed that HSF5 activity plays a crucial role in orchestrating spermatogenesis in mammals, as well as overseeing the events of programmed meiotic sex chromosome remodeling and silencing that occur during meiosis.[77] HSF3 is present in mice but not in humans. HSFX's function is unknown, and HSFY is primarily expressed in the testis and contributes to male fertility.[71] In this review, we shall primarily focus on the HSF1-dependent HSR unless specified otherwise. Nevertheless, we kindly encourage readers to consult the excellent reviews published previously[70,71,73] for additional insights.

HSFs must bind to specific DNA sequences called heat shock elements (HSEs), which are located in the promoter regions of heat shock and other HSF1-driven genes, in order to trigger physiological responses.[74] Canonical HSEs comprise at least three continuous inverted repeats of the pentanucleotide sequence, 5′-nGAAn-3′, alternating between 5′-nGAAn-3′ and 5′-nTTCn-3′, or *vice versa*, where *n* is any nucleotide.[78] Moreover, the varying HSE architecture affects HSF-DNA-binding affinity and the corresponding magnitude of response.[78] The noncanonical (gapped) spacing of nGAAn units in HSE functions to limit the magnitude of transcriptional activation of heat shock genes in response to heat and oxidative stress.[79] This unique feature adds another layer of complexity and refinement to the regulation of HSF1-driven genes.

Mammalian HSFs, particularly the two most studied members of the family, that is, HSF1 and HSF2, exhibit unanticipated complexity in their structure, DNA-binding selectivity, PTMs, interacting partners, and regulation.[71] The distribution of HSEs in the promoter regions of heat shock genes affects the intensity of gene expression as well as the tendency to be activated by different types of stresses. Furthermore, mutations to the HSE are involved in aggregative diseases, such as Huntington's disease.[71]

HSE architecture in heat shock and other HSF1-commanded genes, including features such as proximity to TATA box, number of repeats, and spacing, do determine the speed of gene transcription initiation, sensitivity to heat and other stressors, and the amount of HSP produced in species living in thermally diverse environments.[74,80,81] In addition to specific promoter features, gene properties such as chromatin activation are also required for the activation of heat shock genes.[82,83] Furthermore, HSF1 activity can be suppressed at both the intra- and inter-molecular levels. At the inter-molecular level, molecular chaperones such as HSP70/HSPA, HSP90/HSPC, and TRiC/CCT interact with HSF1 to inhibit its activation, thereby preventing its binding to DNA and regulation of gene expression.[84]

Although the signals that trigger HSF1 activation may vary in their nature, they share a common feature in that such signals typically (but not exclusively) result in elevated levels of misfolded proteins within the cell. However, conditions that can lead proteins to be unfolded, for example, heat and oxidative stress, equally trigger HSF1 activation. In other words, HSF1 can also be activated in *anticipation* of protein denaturation, which is typically a physiological trait always observed during the course of evolution of life.

HSF1 activation can be achieved by at least four known mechanisms, which are not necessarily mutually exclusive.[70] Under normal cellular conditions, HSF1 exists in a complex with cytoplasmic chaperones, specifically DNAJ/HSP40, HSPA/HSP70, and HSPC/HSP90 (mainly).[85] In this state, HSF1 occurs as a monomer without DNA-binding activity. Being part of a multi-chaperone complex, HSC/HSP90 is involved in sequestering HSF1 monomers in the absence of stress and contributes to the deceleration of HSF1 activity after enough HSPs have been induced following stress.[70,86] However, upon exposure to various stresses such as heat shock, inflammation, or unfolded proteins, HSF1 is released from the chaperone complex and translocates into the nucleus. Once in the nucleus, HSF1 undergoes trimerization, hyperphosphorylation, and subsequently binds to HSEs present in HSP genes.[87] Actually, hyperphosphorylation of the regulatory domain of HSF1 is necessary but not sufficient for the full activation of HSF1.[88]

Eventually, the binding of activated HSF1 to HSEs, thus, initiates the transcription of genes encoding HSPs, including HSPA/HSP70 and HSPC/HSP90.[87] This is known as the classical chaperone displacement mechanism. A second possibility is the RNA thermometer model, in which HSF1 remains associated with the heat-sensing RNA molecule HSR-1 under non-stressful situations. Heat stress uncouples HSF1 from the complex at the same time that liberates HSR-1 for binding to the elongation factor eEF1A, thus impeding protein synthesis during the heat shock. A third mechanism is the intrinsic response, in which HSF1 can undertake self-assembly (trimerization) in the presence of heat or other types of stress. Although there may be undiscovered mechanisms for HSF1 self-assembly, oxidation of cysteine residues in the DNA-binding domain of HSF1 is of particular importance because it permits quick formation of disulfide bonds between HSF1 monomers with the consequent trimerization of it,[70] particularly during the course of oxidative stress and metabolic defects in the generation of NADPH (see below).

HSF1-dependent HSR in cells from *Drosophila spp*., vertebrates and in unicellular eukaryotes is *cell-autonomous*,[89] that is, operated within the cell in response to stressful signals addressed to the cell. Nevertheless, a last known mechanism by which HSR and ER-UPR may be activated transcellularly at the organismal level, that is, *cell-nonautonomously* (please see ref.[58]), was initially perceived in *Caenorhabditis elegans*[70] but was now realized to be a mechanism conserved from worms to mammals.[90]

The chaperone machinery involved in the HSR

In order to perform chaperoning of intracellular polypeptides, chaperones definitely do not operate alone. They work in complexes known as *chaperone machines*. The HSPA/HSP70 machine consists of the core HSPA protein along with an array of different cofactors and *nucleotide exchange factors (NEFs)* that transiently bind in cooperation with core HSPA, such as *DNAJ/HSP40*, *HSPH/HSP110, BAG-1*, *Hip*, *HSPBP1, CHIP*, and *HOP*.[52] HSPA1A/HSP70, which is a major work-power for proper protein folding, requires client proteins (substrates) to be presented in order to complete the process, although HSPA1A/HSP70 itself contains the necessary information for folding. This involves necessarily the collaboration of HSPA1A/HSP70 with cochaperones (e.g., HSP40/DNAJ/J-proteins) and several regulatory

cofactors, such as NEFs, or the transfer of client proteins to other chaperones (e.g., HSPC/HSP90) to terminate the process.[64,91,92] NEFs are responsible for promoting the opening of the HSPA/HSP70 nucleotide-binding cleft, which allows for the release of ADP. This, in turn, enables the rebinding of ATP and facilitates the release of substrates from HSPA/HSP70, ultimately promoting efficient chaperone activity.[56] HSPA1A/HSP70 is a notable example where ATP hydrolysis prompts a conformational shift that significantly enhances client-protein affinity.[93]

As noticed by Ritossa in his first studies,[1–3] the HSR may be activated by energy imbalances within cells. However, the integrity of the proteostasis network faces a precarious situation when confronted with energy deficits. This predicament not only triggers the activation of AMPK, which hampers the proteostasis-saving HSR as discussed in the *Introduction* section[16–21] but also precipitates a decline in ATP levels, as expounded upon by Morimoto.[89] This is particularly noteworthy because HSPD/HSP60, HSPA/HSP70, HSPC/HSP90, and HSPH/HSP110 are all dependent on their ATPase activity to facilitate protein folding or refolding.[4] Interestingly, recent evidence shows that HSPH members work as NEFs for HSPA family members.[49,56] Some HSPA/HSP70 homologs, such as the ribosome-associated HSPA14 or the cytosolic HSPH/HSP110 and the ER HSPH4/HSP110 (GRP170) members, which are both homologs of HSPA/HSP70s but act as NEFs for HSPA/HSP70s, show no conservation in the interacting residues.[56]

Chaperones, in addition to facilitating protein folding ("*foldase*" activity), also play a role in the ubiquitin-mediated proteasomal degradation of client proteins when they are irremediably unfolded. The regulation of chaperone-mediated protein degradation is influenced by cochaperones, such as the C-terminal HSP70 binding protein (CHIP). By binding to HSPA/HSP70 and HSPC/HSP90 chaperones via its tetratricopeptide repeat domain, CHIP is able to function as an E3 ubiquitin ligase using a modified RING finger domain (U-box). This unique combination of domains enables CHIP to effectively connect chaperone complexes with the ubiquitin-proteasome system.[94]

Polypeptides in their native form are also transferred from HSP70 to HSP90 chaperones as a means of regulating protein activity. For example, HSP70 and HSP90 jointly control the biological activity of various target proteins through temporary interactions, including but not limited to nuclear receptors (e.g., steroid hormone receptors), kinases (e.g., eIF2α-kinase, cyclin-dependent kinases), and transcription factors (e.g., p53 and HSF1), as well as a diverse array of other proteins.[56] By means of this type of protein-protein interaction, HSPA/HSP70 forms a complex with tumor-related antigens via its polypeptide-binding domain, to elicit greater antigen-specific immune responses.[95] Moreover, HSPs may be involved in the binding of protein fragments from dead malignant cells, to present them to antigen-presenting cells via MHC class I and class II molecules, leading to the activation of anti-tumor $CD8^+$ and $CD4^+$ T cells.

Although not a direct focus of the present discussion, small heat shock proteins (sHSPs/HSPBs) are highly conserved across species and also play a critical role in stress tolerance. Many HSPBs/sHSPs have chaperone-like activity, which helps to prevent the aggregation of target proteins, keeps them in a folding-competent state, and facilitates their refolding either independently or in conjunction with other ATP-dependent chaperones. Mutations in human HSPBs/sHSPs have been linked to myopathies, neuropathies, and cataracts. Moreover, the expression of HSPBs/sHSPs is impaired in various diseases, such as Alzheimer's, Parkinson's, and cancer. The ability of HSPBs/sHSPs to bind Cu^{2+} thereby suppressing the generation of ROS may also have important implications for Cu^{2+} homeostasis and neurodegenerative diseases.[96]

As stated above, the HSR is also intimately connected to inflammation. Accordingly, during either sterile tissue injury or pathogen-elicited Toll-like receptor-2 and receptor-4 triggered inflammatory responses, nuclear factors of κB family (NF-κB) are activated and signal to pro-inflammatory gene expression.[58] On the other hand, a physiological negative feedback system that "resolves" inflammation is also enabled via the HSR pathway. HSF1 may be directly activated by PGE_2-induced rise in temperature (fever) during nuclear factor NF-κB-elicited cyclo-oxygenase-2 induction. PGA_2, the dehydration product of PGE_2, is also able to counteract NF-κB downstream effects by directly blocking IκB kinase-β. HSP70, *per se*, blocks NF-κB activation and transcribing activity.

Following oxidative stress and the formation of reactive oxygen and nitrogen species, ROS/RNS, conformational changes in unfolded proteins can be relayed to HSF1 either directly or via changes in glutathione (GSH)/protein sulfhydryl redox status or, finally, after the activation of the UPR protocol that activates HSF1 through the SIRT1 pathway. This happens through UPR-mediated activation of HuR, an RNA-binding protein that stabilizes SIRT1 mRNA and, consequently, its expression. SIRT1 downstream signals can also be transmitted to the HSR pathway via metabolic alterations, such as those that increase nicotinamide dinucleotide redox status (↑NAD^+/NADH ratio) or adenosine monophosphate to triphosphate (↑AMP/ATP) ratio and the consequent activation of 5′-AMPK. However, under

predominant metabolic stress, activated AMPK impedes the transport of HuR toward the cytosol and may decrease the level of HSF1 activation, thus lessening the HSR and favoring energy preservation. AMPK, the master fuel sensing kinase, may also be turned on by calorie restriction, physical exercise, or the antidiabetic drug metformin, which is also the gold standard for treating metabolic disorders associated with abnormal ovary function, such as polycystic ovary syndrome. Activated AMPK phosphorylates and blocks GSK-3β, which constitutively inhibits HSF1, so that activated AMPK can cause disinhibition of HSF1, leading to an anti-inflammatory HSR. Glutamine metabolism, which is enhanced by muscle contraction, by increasing metabolic flux through the HBP, also blocks GSK-3β thereby enhancing HSR. Estrogen (E2) can activate HSR machinery both directly acting over HSF1 activation and by membrane surface estrogen receptors that signal through Erk1/2, p38 MAPK, and PI3K/Akt pathways leading to enhanced endothelial NO synthase (eNOS) and neuronal NO synthase activity and NO production which, in turn, unveils a discrete redox imbalance that activates HSF1. Additionally, E2 blocks senescence-associated secretory phenotype (SASP) that emanates from continuous activation of NLRP3 (nucleotide-binding and oligomerization domain-like receptor family pyrin domain-containing-3) inflammasome which, in turn, impairs HuR-dependent activation of HSR through the SIRT1-HSF1 pathway. SASP results from long-term ER stress and its consequent unremitted activation of UPR (e.g., obesity, insufficient physical activity). Similarly, HS treatment, even in the fever-like range, blocks NLRP3 inflammasome-dependent SASP, re-establishing HuR-SIRT1-HSF1 downstream pathways, so that HS itself can, paradoxically, re-establish the HSR.[58] Under *predominant proteotoxic stress*, the excess of misfolded proteins leads to the sequestration of protein chaperones (mainly HSP90/HSC) from the associated HSF1 thereby setting this transcription factor free to physically block AMPK. Therefore, in this case, AMPK is shut off and the HSR may be resumed to warrant cellular proteostasis. Please see Figure 2 for an overview of the interplay between the HSR and the resolution of inflammation and confront AMPK in metabolic stress.

It merits note that human immunodeficiency virus-1 (HIV-1) has the ability to effectively co-opt the host cellular machinery, with a particular reliance on the host chaperone machinery for the assembly of its proteins.[97] Among the host proteins involved in this process, HSPBP1, which serves exclusively as a NEF, plays a crucial role in inhibiting HIV-1 long-terminal repeat (LTR) promoter activity. This is because HSPBP1 binds to κB sites at the LTR promoter, which in turn prevents the binding of NF-κB heterodimers (p50/p65) to the same region. Consequently, this leads to the repression of NF-κB-mediated activation of LTR-driven gene expression and HIV-1 proliferation.[98] The same mechanism is thought to take place during the HSR-mediated resolution of inflammation,[58] through the HSPA/HSP70-elicited negative regulation of inflammatory responses through, but not limited to, its negative effect on NF-κB signaling pathway.[8,87,99,100] In the same line of reasoning is the antiviral effect of the antibiotic geldanamycin, which binds to the ATP pocket of HSPC/HSP90, disturbing the binding of HSPC/HSP90 to HSF1 thereby increasing HSPA/HSP70 gene expression. Therefore, HSP90 inhibitors, such as geldanamycin, can activate a strong HSF1-dependent HSR, resulting in elevated levels of HSP70 and HSP90 itself.[97] Importantly, inhibition of HSP90 blocks NLRP3 inflammasome activation,[101] thus preventing inflammasome-mediated SASP and chronic inflammatory diseases, by stimulating HSP70 expression.[58] It has also been suggested drug repositioning with HSPC/HSP90 inhibitors (particularly geldanamycin) for treating COVID-19 patients.[102] Actually, several inhibitors of HSPC/HSP90 have been tested in clinical trials.[103,104] So far, none have reached the clinic, reflecting that inhibition of chaperones is a drastic measure.[53]

With the improvement of experimental tools to identify PTM, it has become clear that not only HSF1, but many chaperones undergo extensive PTMs that determine their chaperone activity as well as interactions of HSP70, HSP90, HSP110, and J-proteins. However, the enzymes responsible for these modifications and the functional consequences that PTMs might have on these proteins remain largely unknown. In addition to the aforementioned transformations and PTM, phosphorylation has notably emerged as an important contributor to the biological activity of HSP70, HSP110, and HSP40/DNAJ interactions. These findings underscore an added layer of complexity in regulating HSP70 function.[105] Lastly, a noteworthy PTM has recently come to light, namely, the covalent modification of HSPs by AMP, a process known as AMPylation. This discovery further reinforces the intricate connection between the HSR and cellular energy levels. When AMPylated, BiP, for instance, exhibits an enhanced capacity for binding and releasing substrates, a diminished basal ATPase activity and a dampened response to ATP hydrolysis stimulation by J-protein cochaperones.[106] These findings compellingly support the notion

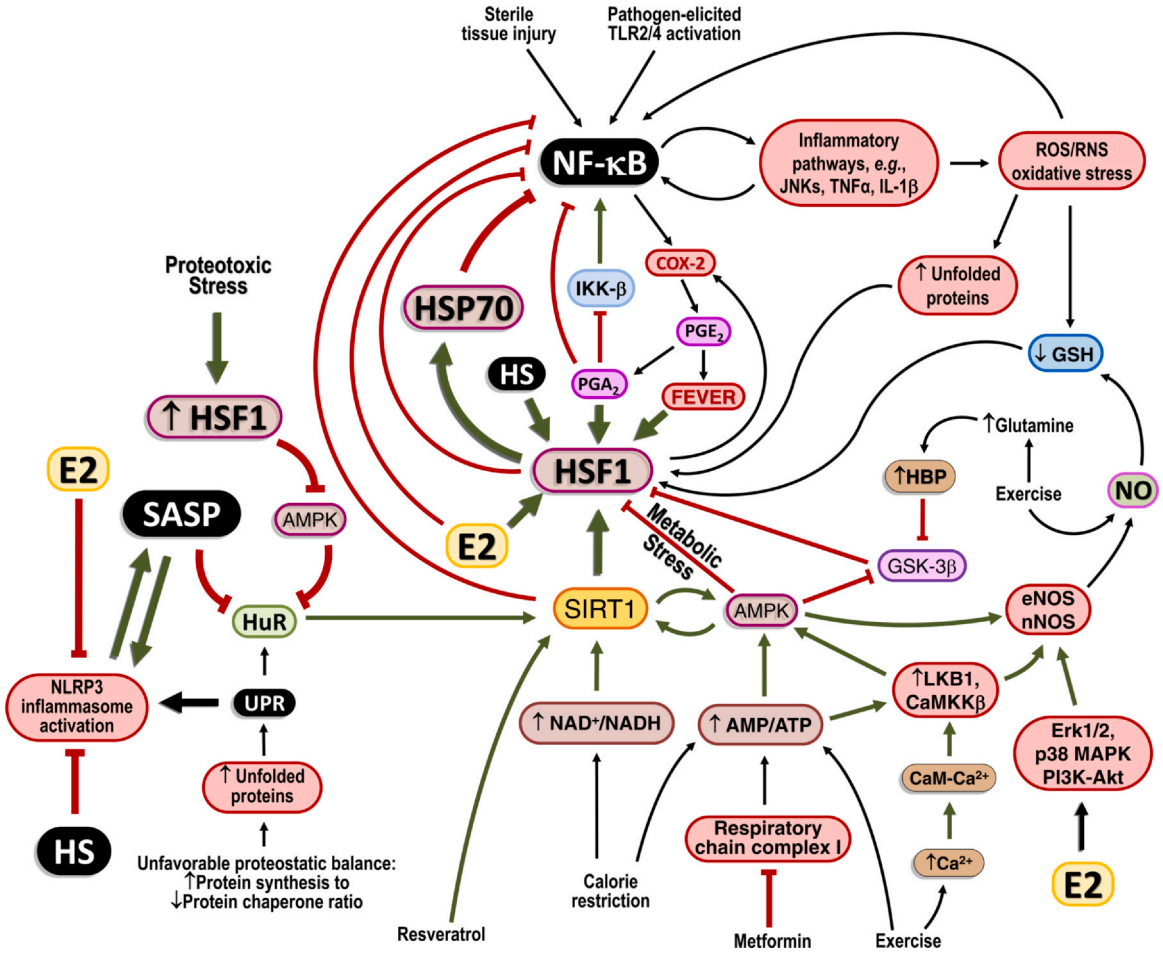


Fig. 2 Overview of how the HSR integrates the interplay between energy sensing (AMPK pathways) and proteotoxic sensing (HSF1 pathways). Stress-activation of the biosynthetic pathway that leads to HSP70 expression from HSF1 couples reproduction (estrogen, E2), exercise, energy balance and proteostasis to anti-inflammation via HSP70. AMPK, 5′-adenosine monophosphate-activated protein kinase; HSP70, the 70 kDa family of heat shock proteins; HSF1, heat shock transcription factor 1; SIRT1, NAD+-dependent deacetylase sirtuin-1; NLRP3, NOD-like receptor pyrin domain-containing protein-3 inflammasome; eNOS, endothelial nitric oxide synthase; ROS, reactive oxygen species.

that AMPylation serves as an inhibitory modification in cellular conditions characterized by reduced demands for anabolic processes or scarcity of energy resources. Kindly refer to Figure 2 for a more comprehensive overview of how the HSR intricately integrates the interplay between energy sensing (AMPK pathways) and proteostasis sensing (HSF1 pathways) defining the regulation of the poise amidst proteotoxic and metabolic stress responses. In any case, it is now evident that both *predominantly metabolic stress* and *predominantly proteotoxic stress* responses are governed by the cellular equivalent of a continuously adjustable rheostat, rather than operating as simple binary on/off switches. These regulatory mechanisms are finely attuned to respond to the swiftly fluctuating states of caloristasis, as postulated by Professor Larry Hightower's group.[24]

Beyond individual chaperone functions discussed here, the concept of *chaperome* analysis is gaining prominence as an approach to understanding the role of the HSR in various conditions, including health and disease. The chaperome comprises a diverse family of proteins, encompassing chaperones, cochaperones, and numerous other factors. When examining chaperome subnetworks in specific contexts such as aging or neurodegenerative diseases, it becomes evident that these subsets serve as protective buffers, preserving proteostasis amid proteotoxic stress.[107] Hence, the current trend is to prioritize the examination of chaperome

networks over the exclusive emphasis on chaperome inhibitors or stimulators, placing them above genetics or client proteins in research priorities.[108]

Coevolution of chaperome and energy-controlling systems

The history of life on Earth, as our present records suggest, was shaped by constant fluctuations in the supply of heat. Since ancient times, Earth experienced numerous and dramatic shifts in its heat dynamics, influencing the adaptation of primordial molecules and the emergence of the first life forms. In the early stages of prebiotic chemistry, energy from the environment was harnessed to construct more complex compounds, enabling the emergence of primordial biochemical systems aimed to protect themselves from processes like hydrolysis and other forms of energy dissipation, approximately 4.0 billion years ago. Notably, during the early Archaean era, Earth's surface temperatures could have reached scorching levels of 80–100 °C, gradually cooling to 30–50 °C around 3.5 billion years ago.[109] Therefore, it comes as no surprise that the biochemical machineries that developed during the Paleoarchean eon, alongside the emergence of the first life forms, evolved to incorporate both heat-preserving and heat-dissipating pathways. This scenario brings to the stage the coevolution of the chaperome and energy-controlling systems which stand as a remarkable testament to life's adaptability and resilience in the face of Earth's ever-shifting thermal challenges.

In contrast to simple prokaryotic organisms, which house thousands of proteins, plants and animals boast an extensive repertoire of hundreds of thousands of proteins. These proteins exhibit greater length, possess multiple functional domains, and encompass a wide array of intricate folds and combinations, interspersed with repeated segments and beta-rich architectures that render them susceptible to misfolding and aggregation.[4] Interestingly, the relative representation of core chaperones responsible for upholding the fidelity of protein folding in increasingly intricate proteomes underwent minimal change from prokaryotic to mammalian genomes.[4] This observation is not unexpected, given the pivotal role of protein chaperoning in sustaining cellular vitality. Thus, the optimal evolutionary solution that originated in the LUCA, predating the schism between archaea and bacteria, has persevered largely unaltered since that epoch. Only marginal adaptations have been introduced to accommodate the escalating complexity of proteins over time.

Evidence suggests that the genesis of core chaperones can be traced back to the early diverging prokaryotes, marking the advent of HSP60 as the first core chaperone. HSP60, an ATP-powered unfoldase possessing a cage-like structure, emerged more than 3.6 billion years ago during the Paleoarchean eon, within the era of LUCA. Concurrently, the appearance of HSP20/GroES anti-aggregation chaperones may have occurred in tandem with HSP60 during this epoch. In contrast, the introduction of HSP70, HSP90, and HSP100 into the bacterial domain likely transpired around 3.25 billion years ago. Subsequently, the emergence of chaperones such as HSP110, Bag Hop, and other cochaperones unfolded within the Proterozoic era, around 2 billion years ago, during the era of the last eukaryotic common ancestor, as highlighted by Rebeaud and colleagues[4] in their recent but seminal paper.

Key milestones in microbial evolution were influenced by critical events, including the extensive glaciation of the late Neoproterozoic Snowball Earth, approximately 750–580 million years ago, and the subsequent oxygenation event of the oceans and atmosphere approximately 630–551 million years ago. These events set the stage for the explosion of complex life forms in the Cambrian, about 540–520 million years ago.[110] Particularly noteworthy is the Neoproterozoic oxygenation event (around 600 million years ago), a turning point when metazoans, and subsequently chordates, emerged and faced the imperative necessity to produce protective chaperones against oxidative stress that became pronounced.

In a manner akin to the discussed chaperome, reconstructions suggest a systematic transfer of sequences encoding glycolytic enzymes among diverse organisms. Similar to chaperones, there appears to be limited exchange between bacterial and eukaryotic domains.[111] Driven by negative selection and subtle enzymatic alterations, significant evolutionary shifts in the function of ADP-dependent sugar kinases conferred vital adaptive advantages, such as enhancing glycolytic efficiency in archaea roughly 3.594 billion years ago.[5] Apropos, *ADP*-dependent sugar kinases stand out for their distinctive feature of phosphorylating glucose and fructose-6-phosphate using ADP, instead of ATP as the phosphoryl donor. They appear to represent not the last ancestral forms but rather a transitional link between glucokinase and phosphofructokinase found in modern archaea and higher eukaryotes alike.[112]

Glycolytic enzymes, being ubiquitously present, have been instrumental in deciphering evolutionary relationships between organisms. These enzymes serve as suitable evolutionary chronometers, exhibiting a rate of change slow enough to discern broad evolutionary patterns yet swift enough for precise classification of closely related organisms.[113] The phylogenetic divergence of *sugar*

kinases and *chaperones* traces back to their respective common ancestors approximately 3.5 billion years ago.[5] Beyond their roles in cellular energy production, notably exemplified by hexokinase as the primary enzyme initiating intracellular glucose metabolism, nature seems to have intricately interwoven sugar metabolism and proteostasis protection within a singular molecule: HSP70. Notably, during the elucidation of the three-dimensional structure of the ATPase fragment of HSPA8 (HSC70), Flaherty and colleagues[6] serendipitously uncovered striking structural (spatial) similarities, particularly in subdomains Ia and IIa of HSC70 (Figure 3), between the chaperone and hexokinase, despite disparities in their amino acid sequences. Furthermore, significant structural resemblances were found with other sugar kinases, including fucokinase, gluconokinase, xylulokinase, ribulokinase, and glycerokinase. These kinases are predicted to share subdomains with a comparable tertiary structure to the ATPase subdomains Ia and IIa of hexokinase (Figure 3), actin, and HSC70, exhibiting a similar ATP-binding pocket and the ability to undergo interdomain hinge motion during functional state transitions.[7]

Importantly, the divergence among sugar kinases, actin, and HSC70 predates the prokaryote-eukaryote divergence, as HSC70 family members are found in both eukaryotic and prokaryotic organisms.[7] This suspicion is further corroborated by conformational changes occurring within the ATPase domain that facilitate nucleotide-controlled communication with the substrate-binding domain of HSP70. Evidence indicates that the nucleotide-binding cleft undergoes opening and closure dynamics during the nucleotide-binding/release cycle of both HSP70 and hexokinase.[114,115] A pivotal mechanism underlying HSP70 allostery involves the transmission of information from the nucleotide-binding site to the interdomain linker. In the presence of ATP, the linker interacts with the periphery of the IIa β-sheet, establishing a structural connection between the linker and the nucleotide-binding site. Consequently, an allosteric communication pathway is established, facilitating the transmission of signals from the NBD to the substrate-binding domain via the interdomain linker.[116,117]

It is intriguing that a 16 kDa nucleoside diphosphate (NDP) kinase, p16, from the Nm23/NDP kinase family, serves as an accessory protein to HSPA8 (HSC70). This protein can influence HSPA8's oligomeric state and dissociate unfolded proteins from HSPA8 even in the absence of exogenous ATP.[118] NDP kinases facilitate the exchange of terminal phosphates between different NDPs and triphosphates in a reversible manner, resulting in the production of nucleotide triphosphates. Interestingly, p16 is also stress-responsive and exhibits higher sensitivity to proteotoxic stress compared to

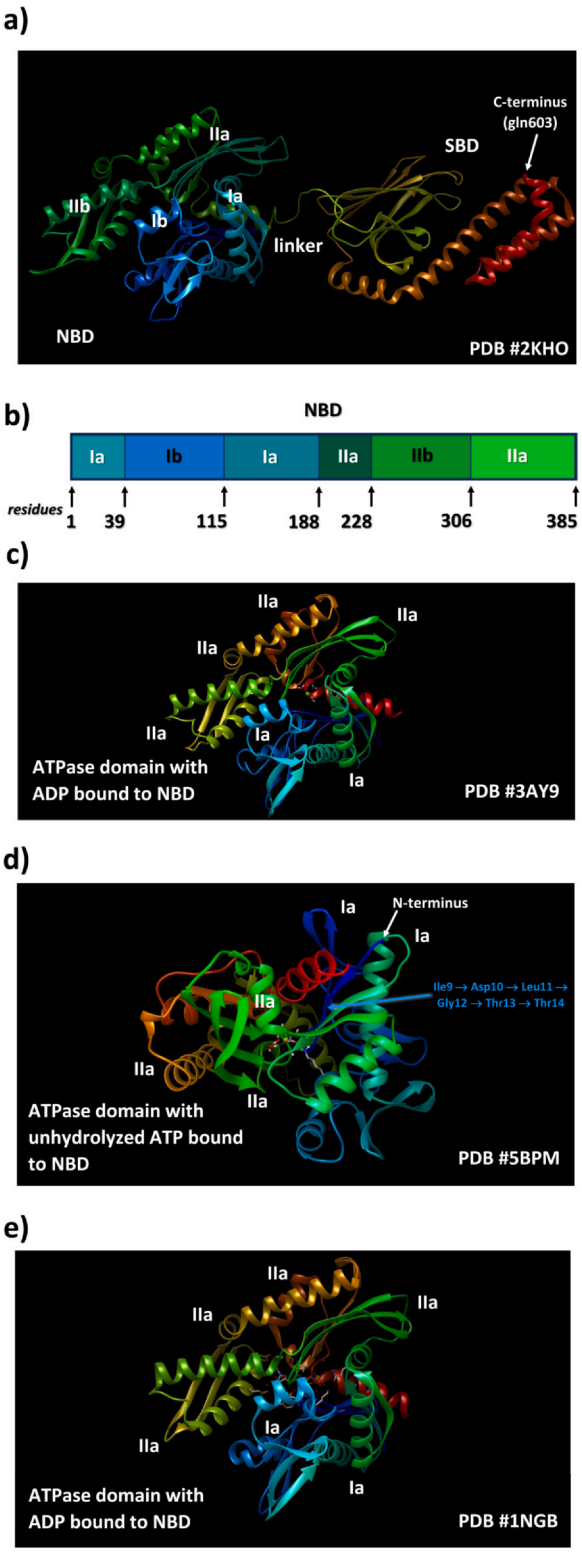


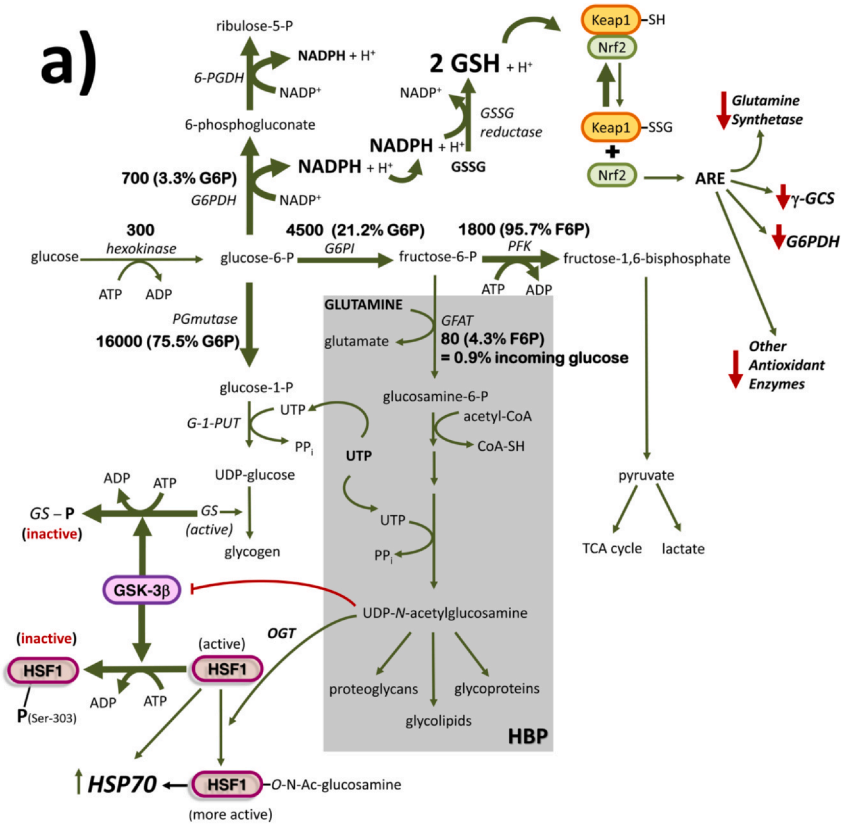
Fig. 3 Representative structures of HSP70. (a) Nuclear magnetic resonance/residual dipolar couplings/X-ray resolution of the tridimensional structure of prokaryotic HSP70 (*Escherichia coli*'s DnaK) in complex with ADP and a peptide (PDB #2KHO, residues 1-605) showing hexokinase subdomains (Ia and IIa) in the 45 kDa nucleotide-binding domain (NBD). The 30 kDa substrate-binding domain (SBD) is also given. (b) Spatial

disposition of Ia and IIa (hexokinase subdomains of ATPase domain) and Ib and IIb (non-hexokinase subdomains) along with their respective residues on NBD of HSP70. (c) X-ray diffraction-solved structure of human HSP70 (HSPA1A/1B) NBD (PDB # 3AY9) in the ADP-bound conformation. (d) X-ray diffraction-solved structure of human HSP70 (HSPA1A) with unhydrolyzed ATP bound to NBD (PDB #5BPM). The N-terminal sequence (Ile9 - Asp10 - Leu11 - Gly12 - Thr13 - Thr14), which is highly conserved among all HSP70s, is depicted in the dark-blue ribbon. (e) X-ray diffraction-solved structure of bovine HSC70 (HSPA8) in the ADP-bound conformation (PDB #1NGB). Structure files were obtained from RCSB Protein Data Bank (PDB) at https://www.rcsb.org/structure/.

HSC70 or HSP70 themselves. By converting HSPA8 oligomers into active monomers, p16 appears to regulate HSPA8 activity. Given that ATP/ADP exchange occurs slowly, and no homologs of the Gro-P like protein E NEF have been discovered in the cytoplasm, some substrates may be released very slowly without assistance from accessory proteins. In the presence of p16, however, substrates can be released from HSC70-ADP, and subsequent ATP/ADP exchange transforms HSC70 into a form that is most accessible for substrate binding, as described by Leung and Hightower in 1997.[118] Moreover and importantly, phylogenetic analyses of NDP kinases have revealed that these kinases also share a common ancestor in LUCA, as demonstrated.[119] This parallels what has been observed with chaperones and sugar kinases. Therefore, it is not unreasonable to consider that an ancestral form of p16 NDP kinase might have played a role in the evolutionary development of early ATP-fueled chaperones, providing an initial link between HSPA8/HSPA1A and energy metabolism. Notably, rat HSC70 proteins expressed in *E. coli* exhibit significant structural similarities with *E. coli* DnaK protein,[120] a much older protein in the Tree of Life. This may suggest that an ancestral ATPase activity may still be present in HSPA8, reinforcing the connection between chaperones and energy metabolism. Considering all the above observations, therefore, it is plausible that the 44 kDa ATPase fragment of present-day HSC70 had evolved from a common ancestor shared with hexokinases and actin. The question of whether HSC70 possesses some kinase activity toward glucose, however, remains a subject of debate, yet it warrants investigation.

In addition to the role of AMPK, which can either downregulate or upregulate the HSR through the GSK-3β/HSF1 duet, as previously discussed, another pathway involving glucose metabolism, known as the HBP, plays a significant role in HSR regulation. The HBP, which is a nutrient-sensing pathway with intricate ties to energy metabolism and the HSR, extends beyond the role of GSK-3β in glycogen synthesis.[15,121,122] Elevated levels of circulating glutamine, as seen during exercise, lead to a notable diversion of fructose-6-phosphate from glycolysis into the HBP through its interaction with glutamine, catalyzed by GFAT (glutamine-fructose-6-phosphate transaminase). This results in the production of glucosamine-6-phosphate. The final metabolite of the HBP, UDP-*N*-acetylglucosamine (UDP-GlcNAc), exerts a dual effect on the HSR: firstly, by inhibiting GSK-3β and secondly, by covalently modifying HSF1. This modification involves *O*-linked *N*-acetylglucosaminylation, enhancing HSF1's DNA-binding and transcriptional activities regarding heat shock genes. The heightened availability of glutamine stimulates increased flux through the HBP, resulting in elevated HSP70 expression.[123] A comparison between parts A and B of Figure 4 illustrates the flux balance analysis of the HBP[15] under two distinct plasma and muscle glutamine concentrations in rats.[124] Additionally, the intensified flux through the HBP induces a minor redox imbalance, sufficient to upregulate the expression of genes responsible for redox protection, including those involved in the biosynthesis of GSH and glutamine itself.[15,124,125] *De novo* GSH synthesis primarily occurs through transcriptional regulation, involving a series of signaling events culminating in the binding of nuclear factor-erythroid 2 p45-related factor 2 to promoter regions containing antioxidant response elements within the nucleus.[126–131] These findings highlight the intimate interplay between the HSR, energy metabolism, and redox protection (crucial for proteostasis). They also shed light on why glutamine emerges as a potent co-inducer of the HSR, that is, HFS1 must be previously recruited to have its function augmented by HBP. Please see Figure 5 for an integrative overview of caloristasis networks and energy regulation through glucose metabolism.

The HSR has not only evolved at the molecular level but also at the organismal level, forming a close linkage with the sympathetic nervous system (SNS), which serves as the principal physiological sentinel responsible for safeguarding homeostasis against *all* forms of external threats. As expected, the activation of the SNS in situations such as starvation, physical exercise (fight-or-flight stress), and even heat therapy itself[11,132] can elicit a potent and sustained HSR.[58] Indeed, the intricate connections among glucose levels, energy sensing, the HSR, and sympathetic activity converge within the ventromedial hypothalamus (VMH), a central hub orchestrating functions like feeding, fear, thermoregulation, and sexual activity. The VMH also serves as a site for a robust estrogen-induced HSP70 response.[133,134] Furthermore, norepinephrine can modulate the hypothalamic mechanisms responsible for fever induction,


a)
ribulose-5-P
6-PGDH
NADPH + H+
NADP+
6-phosphogluconate
2 GSH + H+
NADP+
GSSG reductase
NADPH + H+
GSSG
700 (3.3% G6P)
G6PDH
NADPH + H+
NADP+
Keap1 –SH
Nrf2
Keap1 –SSG
Nrf2
ARE
Glutamine Synthetase
γ-GCS
G6PDH
Other Antioxidant Enzymes
300
glucose
hexokinase
ATP ADP
glucose-6-P
4500 (21.2% G6P)
G6PI
fructose-6-P
1800 (95.7% F6P)
PFK
ATP ADP
fructose-1,6-bisphosphate
PGmutase
16000 (75.5% G6P)
GLUTAMINE
glutamate
GFAT
80 (4.3% F6P)
= 0.9% incoming glucose
glucose-1-P
G-1-PUT
UTP
PPi
glucosamine-6-P
acetyl-CoA
CoA-SH
UTP
ADP ATP
UDP-glucose
GS – P
(inactive)
GS
(active)
glycogen
UTP
PPi
pyruvate
TCA cycle
lactate
GSK-3β
UDP-N-acetylglucosamine
(inactive)
HSF1
P(Ser-303)
ADP ATP
(active)
HSF1
OGT
proteoglycans
glycoproteins
glycolipids
HBP
HSP70
HSF1
O-N-Ac-glucosamine
(more active)

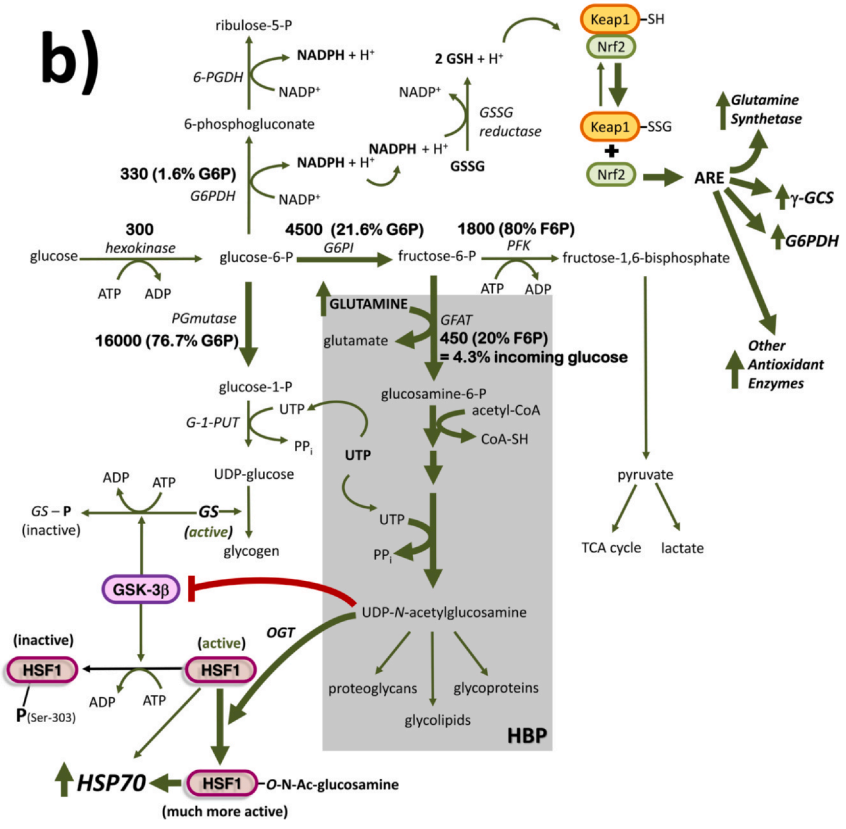

b)
ribulose-5-P
6-PGDH
NADPH + H+
NADP+
6-phosphogluconate
2 GSH + H+
NADP+
GSSG reductase
NADPH + H+
GSSG
330 (1.6% G6P)
G6PDH
NADPH + H+
NADP+
Keap1 –SH
Nrf2
Keap1 –SSG
Nrf2
ARE
Glutamine Synthetase
γ-GCS
G6PDH
Other Antioxidant Enzymes
300
glucose
hexokinase
ATP ADP
glucose-6-P
4500 (21.6% G6P)
G6PI
fructose-6-P
1800 (80% F6P)
PFK
ATP ADP
fructose-1,6-bisphosphate
PGmutase
16000 (76.7% G6P)
GLUTAMINE
glutamate
GFAT
450 (20% F6P)
= 4.3% incoming glucose
glucose-1-P
G-1-PUT
UTP
PPi
glucosamine-6-P
acetyl-CoA
CoA-SH
UTP
ADP ATP
UDP-glucose
GS – P
(inactive)
GS
(active)
glycogen
UTP
PPi
pyruvate
TCA cycle
lactate
GSK-3β
UDP-N-acetylglucosamine
(inactive)
HSF1
P(Ser-303)
ADP ATP
(active)
HSF1
OGT
proteoglycans
glycoproteins
glycolipids
HBP
HSP70
HSF1
O-N-Ac-glucosamine
(much more active)

Fig. 4 Heat shock response interplay with glutamine metabolism via hexosamine biosynthetic pathway (HBP). Depicted are the major routes of glucose utilization after its entry into cells. Soon after passing the hexokinase (HK) bottleneck, phosphorylated glucose may be diverted to glycolysis, glycogen synthesis, or the pentose-phosphate shunt (hexose-monophosphate shunt), in a proportion that depends on the cell type and physiological conditions. The present artwork is a graphic illustration of experimental values obtained from soleus and gastrocnemius muscles of 8-week trained (treadmill) rats treated or not with L-glutamine supplementations during the last 21 days.[124] Under L-glutamine supplementations, excess intramuscular L-glutamine supply enforces fructose-6-phosphate (F6P) to divert from glycolysis and enter the *HBP* (shaded box in the center) thus changing the heat shock response and antioxidant metabolism. The thicknesses of the arrows indicate the approximate proportion of each metabolite entering each given sub-pathway (parentheses). Data are given in $\mu mol\ min^{-1}$ mg $protein^{-1}$. ARE, antioxidant response elements; HSF1, heat shock transcription factor 1.
Reused from: Physiological regulation of the heat shock response by glutamine: implications for chronic low-grade inflammatory diseases in age-related conditions. *Nutrire*. 2016;41:17. https://doi.org/10.1186/s41110-016-0021-y. Under the open access Creative Common CC BY license 4.0.

an HSR stimulator, particularly in the preoptic area of the hypothalamus.[135] It is noteworthy that acute hypoglycemia (metabolic stress) triggers the release of extracellular HSP72 and pro-inflammatory cytokines, such as IL-6.[136] Contrarily, glucose ingestion inhibits exercise-induced eHSP70 secretion[137] and abolishes the counterregulatory responses prompted by hypoglycemia via the SNS.[138] This modulation is reliant on the neuronal circuitry of VMH.[139]

While HSPA/HSP70 chaperones are abundant and extensively studied as a major work-force of core chaperones, HSPC/HSP90 plays a crucial role in the

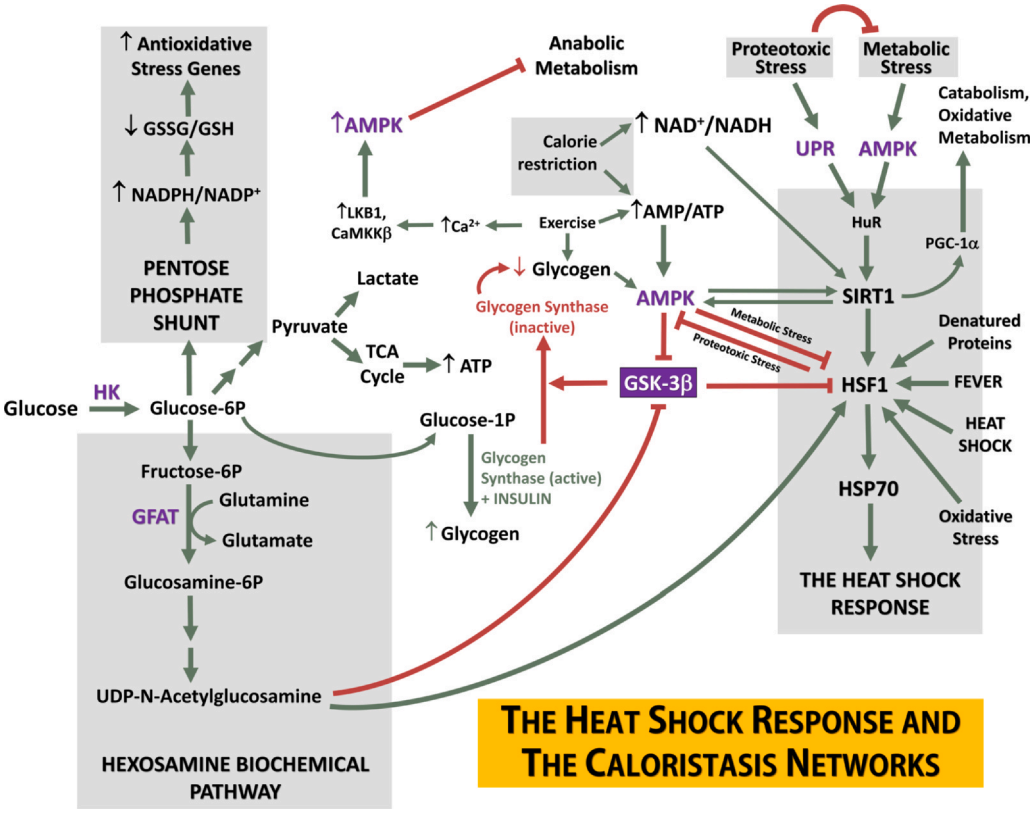


Fig. 5 Extended perspective on the heat shock response within the context of caloristasis. An illustration of potential alterations in glucose metabolism, diverging from the typical predominance of cytosolic glycolysis, is given. These deviations can lead glucose metabolism towards two critical pathways: the pentose-phosphate shunt, involved in lipid synthesis and antioxidant metabolism, or the hexosamine biochemical pathway, which modulates glycogen synthase-3β and, consequently, impacts HSF1 activity and the regulation of the heat shock response itself. The pivotal role of AMP-activated protein kinase (AMPK) in this process is highlighted. HSP70, the 70 kDa family of heat shock proteins; HSF1, heat shock transcription factor 1; HuR, human antigen R, a.k.a. ELAV-1, for embryonic lethal, abnormal vision, Drosophila, homolog-like protein-1; SIRT1, NAD^+-dependent deacetylase sirtuin-1; UPR, unfolded protein response.

cardiovascular system by binding to key proteins involved in vascular relaxation, such as endothelial nitric oxide synthase (eNOS/*NOS3*) and soluble guanylate cyclase.[140] Moreover, nitric oxide generated in blood vessels by eNOS serves as a potent inducer of HSP70 synthesis. This induction occurs because nitric oxide induces slight oxidative stress, activating HSF1 via disulfide bond formation.[11,70] Besides this, HSPC/HSP90 also chaperones essential proteins related to animal reproduction, including glucocorticoid receptor, progesterone receptor, estrogen receptor, androgen receptor, mineralocorticoid receptor, and HSF1 itself.[85,141]

The interplay between reproduction, HSR, and energy control goes beyond and reveals how evolution brought together physiological functions that depend on energy perception and the HSR. Accordingly, a primary clue to this understanding comes from the interactions between steroid hormone receptors and HSPC/HSP90, which are instrumental in guiding steroid hormones to their specific target promoters. However, further away from their role as regulators of steroid hormone receptors, steroid hormones themselves play a pivotal role in governing a critical facet of the HSR that pertains to reproduction in mammals and birds. As reviewed by Miragem and Homem de Bittencourt,[11] E2 not only influences the function of steroid hormone receptors but also initiates HSR in hypothalamic neurons responsible for producing gonadotropin-releasing hormone (GnRH). In this context, GnRH neurons release GnRH into the median eminence, a process that triggers the secretion of luteinizing hormone by the pituitary gland and, consequently, induces ovulation/estrus. However, for the secretion of GnRH to occur, GnRH neurons depend on input from kisspeptin-neurokinin B-dynorphin (KNDy) neurons located in the infundibular nucleus (arcuate nucleus in other mammals). KNDy neurons, which dictate the rhythm and intensity of pulsatile GnRH secretion, are responsive to various factors, including temperature, prostaglandin E_2 (PGE_2) during immune responses, energy reserves signaled by adipokines, and the overall metabolic status as conveyed by the VMH through medial preoptic and median preoptic nuclei of the hypothalamus. Lastly, VMH serves as the trigger for activating SNS efferences in response to perceived threats to homeostasis. These inputs collectively regulate the pulsatile secretion of GnRH, ultimately leading to ovulation/estrus and heightened E2 production. Furthermore, E2, in turn, acts as a potent inducer of HSP70 expression in various regions of the hypothalamus that are involved in both reproduction and energy sensing. Please refer to Figure 2 for a schematic representation of the role of E2 within the HSR.

The influence of environmental signals on reproduction involving the HSR extends beyond humans and mammals. In the avian realm, for instance, gonadotropin (follicle-stimulating hormone and luteinizing hormone) synthesis and release are stimulated by GnRH, as observed in breeding birds.[142] Seasonal breeding avian species further showcase the intricate interplay between environmental factors such as light, temperature, food availability, and mate presence, all meticulously integrated by HSR-eliciting KNDy neurons to regulate reproduction, by modulating the release of gonadotropins from the pituitary gland.[11] A practical example of this connection can be witnessed in poultry farming: anyone who raises chickens knows that, when the weather gets excessively hot (surplus energy) or cold (scarce energy availability), or when the chicken is too fat (excess energy storages) or too lean (low energy reserves), egg production takes a significant hit. Seasonal breeders like sheep exhibit reproductive quiescence during the spring and early summer months (hotter weather). The measurement of circannual time crucially relies on photoperiod changes, with the pineal gland assuming a pivotal role by secreting melatonin during the night. This translates environmental cues into physiological signals that find integration at the level of HSR-elaborating KNDy neurons.[143] In essence, the E2-sensitive KNDy neurons not only serve as conduits for integrating environmental energy signals into the territory of reproduction but also play a dual role by triggering the HSR, a cellular mechanism providing anti-inflammatory and proteostasis-saving functions. This allows for E2-mediated protection against neurodegenerative diseases during the reproductive phase of vertebrates. Therefore, animal reproduction and neuroprotection are dependent on energy sensing that triggers the cytoprotective HSR.

For quite some time, it has been established that heat plays a pivotal role in shaping various metabolic adaptations. For instance, in cold-acclimated trout exposed to temperatures as low as 4 °C, there is a redirection of glucose utilization. This shift leads to an enhancement in the rate of fat synthesis and promotes a more efficient energy production system, particularly through fat oxidation, as part of the organismal response to cold compensation.[144] Furthermore, evolution has masterminded adjustments within biochemical systems to ensure the preservation of fundamental structures and processes across diverse organisms, irrespective of their environmental milieu.[145]

Much like the arguments in the *Introduction* section regarding the stenothermal Antarctic fish Emerald

rockcod,[44] stenothermal Antarctic notothenioid fish populations exhibit deficiencies in their capacity for temperature-induced gene expression, notably the absence of a genuine HSR. These limitations may impair their ability to acclimate to elevated temperatures, with these animals remaining unable to acclimate to temperatures exceeding approximately 4 °C. Curiously, full HSR is observed in temperate New Zealand notothenioids, suggesting that the evolutionary adaptation to cold and stable thermal conditions has depleted the genetic resources of Antarctic fishes.[146] Conversely, the preservation of a robust HSR equips exotic invader species with the means to acclimate to new environments and potentially outperform their native counterparts in metabolic adaptations.[147]

In light of the broader insights into the evolution of the chaperome and sugar kinases, a remarkable convergence emerges—both in the context of heat exchange between environmental factors and molecular entities, and subsequently, between the environment and cellular systems responsible for energy production and protection against proteotoxic stresses. This convergence becomes especially pronounced upon considering that AMPK, the central regulator of cellular energy sensing, features a glycogen-binding domain on its β regulatory subunit,[148] endowing it with the capacity to function as a glycogen sensor. Remarkably, it is of note that glucose can downregulate AMPK through protein phosphatase 2A, a phenomenon observed in various contexts, including rodent islets, β-cell strains,[149] and yeast.[150] This underscores the complexity of cellular responses to metabolic stress, which may hinge upon the intricate proteostasis of the cells. When cells confront another type of metabolic stress, suboptimal oxygen conditions, not only does total ATP turnover significantly decrease, but each ATP-consuming process experiences distinct impacts. In essence, only vital processes, such as the sodium/potassium pump necessary for maintaining membrane potential and cell volume, assume prominence as the primary energy sinks in the energy-restricted state.[151] Similarly, in the context of hyperbaric oxygen therapy, as demonstrated in an elegant set of experiments by Tezgin and co-workers, application of high partial pressure of oxygen redirects mitochondrial metabolism towards cytosolic glycolysis, that is, the Warburg effect also known as aerobic glycolysis,[152] serving as a means to maintain proteostasis without necessitating the activation of a conventional HSR.[24] Similarly and notably, nutrient deprivation induces the generation of proteotoxic ROS alongside AMPK-dependent phosphorylation of pyruvate kinase.[153] This phosphorylation inhibits pyruvate production, forcing cells to rely solely on cytosolic glycolysis, ultimately resulting in the Warburg effect, where lactate accumulates. Consequently, it is reasonable to anticipate that evolution has minimized the ATP-consuming chaperone machinery in such states, thereby justifying the discussed caloristatic control switch (Figure 1).

Conclusion

In the grand tapestry of cellular life, the evolution of the HSR and the emergence of sugar kinases appear as two threads, interwoven over billions of years, each influencing the other in a dance of molecular intricacy. Just as nature crafts a mosaic of elements into a harmonious whole, the HSR, originally designed to combat proteotoxic challenges, now stands as a sentinel guarding the sanctity of cellular energy metabolism. Actually, the HSR is a story of sentinels; sentinels of homeostasis. Professor Ferruccio Ritossa's pioneering insights revealed that the HSR, like a sentinel, senses not only the denaturing fires of heat but also the flickering uncertainties of energy paucity, working as a sentinel ready to rise in defense against both. This revelation, echoing through the annals of scientific discovery, aligns the HSR as a guardian of cellular homeostasis, a shield against the chaos that threatens proteostasis and metabolic equilibrium.

As we trace the evolutionary footprints left by the LUCA, a disclosure emerges—the origins of the proteostasis-defending systems coincide with the nascent steps of sugar kinases, as if destiny bound them together in the crucible of time. The NBD of HSP70, the main work-power of the protein chaperone machinery, bears subdomains akin to those found in sugar kinases, a testament to the ancient partnership between protein folding and energy production.

Beyond the realm of molecular intricacies, the HSR extends its embrace to the wider landscape of cellular life, integrating energy metabolism and the resolution of inflammation. In a delicate choreography, AMPK, the sentinel of energy scarcity, assumes the mantle of a maestro, orchestrating a symphony of responses that can vary from energy-saving, proteostasis-protecting or resolution of inflammation. Meanwhile, SIRT1, awakened by the whispers of calorie restriction, joins the orchestra, amplifying the HSR's melody. This interplay extends beyond chaperones, shaping the destiny of energy metabolism genes themselves. As glucose-regulated chaperones harmonize their tune with the HSR, the HBP adds its notes to the score, guiding GSK-3β to

silence and HSF1 to rise. Yet, within this symphony, paradoxes arise, akin to a melody that shifts unexpectedly. AMPK, usually an ally in the HSR's anti-inflammatory cause, becomes an enigmatic muse, impeding the nuclear journey of HuR, the stabilizer of SIRT1 mRNA. This paradoxical dance reveals the nuanced equilibrium of cellular stress responses, a dance governed by the dual maestros, AMPK and HSF1, creating a new composition—caloristasis, a delicate thermodynamic balance between energy homeostasis and proteostasis, woven with precision.

In this intricate mosaic, our goal was to shed light on the interplay between the HSR as a guardian of proteostasis and the pathways dictating energy metabolism. The caloristatic controlling switch, summarized in Figure 1, and in an extended view in Figures 2 and 5, may hold the key to unraveling the mysteries of obesity, diabetes, cardiovascular and neurodegenerative diseases, reproductive abnormalities, as well as pharmacological and non-pharmacological interventions including the artistry of well-structured exercise routines. Like a maestro conducting an orchestra, we stand on the precipice of discovery, ready to uncover the hidden harmonies that govern life's symphony.

Funding and support This work has been supported by The State of Rio Grande do Sul Foundation for Research Support (FAPERGS/Decit/SCTIE/MS/CNPq/SESRS n. 03/2017-PPSUS #17/2551-0001424-3 to MSK and #19/2551-0001713-8 to PIHBJ) and The Brazilian National Council for Scientific and Technological Development (CNPq, process #303853/2017-4 to PIHBJ, process #307926/2022-2 to TGH and process # 302959/2020-3 to MSK). Financial support from CAPES (Coordination of Superior Level Staff Improvement) is also acknowledged.

Author contribution PIHBJ conceptualized the paper, authored the initial draft, and oversaw its finalization. All the authors were involved in co-writing this work. PIHBJ prepared the figures. All the authors have read and agreed to the submitted and published versions of the manuscript.

Data availability statement Data will be made available on request.

Declarations of interest The authors declare no conflict of interest and no competing interests such as consultancies, financial involvement, patent ownership, etc. in relation to the work described. CNPq, FAPERGS, and CAPES (the funding organisms) had no involvement in the propositions presented in this manuscript.

Acknowledgments The authors are indebted to Dr Maria Inês Lavina Rodrigues for her invaluable technical support during the experiments that support many of the findings described herein. We apologize for not including many important primary studies in our text due to space constraints. Our goal was to ensure a smooth flow of information throughout the text.

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Capítulo II

Título: Resolution of inflammation in chronic disease via restoration of the heat shock response (HSR)

Periódico: Cell Stress and Chaperones

Situação: Publicado em fevereiro de 2024.

(disponível em: <https://doi.org/10.1016/j.cstres.2024.01.002>).

Cell Stress and Chaperones (2024) 29:116–142
https://doi.org/10.1016/j.cstres.2024.01.002

REVIEW

Heat shock response during the resolution of inflammation and its progressive suppression in chronic-degenerative inflammatory diseases

Helena Trevisan Schroeder[1,a] · Carlos Henrique De Lemos Muller[2,b] · Thiago Gomes Heck[3,c] · Mauricio Krause[2,d] · Paulo Ivo Homem de Bittencourt[1,e,*]

Received: 18 September 2023 / Revised: 11 January 2024 / Accepted: 12 January 2024

Abstract

The heat shock response (HSR) is a crucial biochemical pathway that orchestrates the resolution of inflammation, primarily under proteotoxic stress conditions. This process hinges on the upregulation of heat shock proteins (HSPs) and other chaperones, notably the 70 kDa family of heat shock proteins, under the command of the heat shock transcription factor-1. However, in the context of chronic degenerative disorders characterized by persistent low-grade inflammation (such as insulin resistance, obesity, type 2 diabetes, nonalcoholic fatty liver disease, and cardiovascular diseases) a gradual suppression of the HSR does occur. This work delves into the mechanisms behind this phenomenon. It explores how the Western diet and sedentary lifestyle, culminating in the endoplasmic reticulum stress within adipose tissue cells, trigger a cascade of events. This cascade includes the unfolded protein response and activation of the NOD-like receptor pyrin domain-containing protein-3 inflammasome, leading to the emergence of the senescence-associated secretory phenotype and the propagation of inflammation throughout the body. Notably, the activation of the NOD-like receptor pyrin domain-containing protein-3 inflammasome not only

Abbreviations: AMPK, 5'-adenosine monophosphate-activated protein kinase; ATF6, activating transcription factor 6; COVID-19, coronavirus disease-2019; COX, cyclooxygenase (prostaglandin endoperoxide H synthase); CRP, C-reactive protein; CVD, cardiovascular disease(s); ER, endoplasmic reticulum; GSK-3β, glycogen synthase kinase-3β; HBP, hexosamine biosynthetic pathway; HFD, high-fat diet; HSF1, heat shock transcription factor-1; HSP, heat shock protein; HSP70, the 70 kDa family of heat shock proteins; eHSP70, extracellular HSP70; HSR, heat shock response; HuR, human antigen R, a.k.a. ELAV-1, for Embryonic Lethal, Abnormal Vision, Drosophila, Homolog-Like protein-1; IAPP, pancreatic islet amyloid polypeptide; IRE1α, Inositol Requiring Enzyme-1, also known as Endoplasmic Reticulum-to-Nucleus Signaling-1, ERN1; IκB, inhibitors of κB transcription factors; JNK, c-Jun N-terminal kinase; NF-κB, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; NLRP3, NOD-like receptor pyrin domain-containing protein-3 inflammasome; NOD, nucleotide-binding oligomerization domain; PGs, prostaglandins; cyPG, cyclopentenone prostaglandin; PERK, Protein kinase-like ER Kinase; PP2A, protein phosphatase 2A; SASP, Senescence-Associated Secretory Phenotype; SIRT1, NAD^+-dependent deacetylase sirtuin-1; SNS, sympathetic nervous system; TCS, transcellular chaperone signaling; T2DM, type 2 diabetes mellitus; TNFα, tumor necrosis factor-α; UPR, unfolded protein response

* Paulo Ivo Homem de Bittencourt
pauloivo@ufrgs.br

[1] Laboratory of Cellular Physiology (FisCel), Department of Physiology, Institute of Basic Health Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil

[2] Laboratory of Inflammation, Metabolism and Exercise Research (LAPIMEX), Department of Physiology, ICBS, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil

[3] Post Graduate Program in Integral Health Care (PPGAIS-UNIJUÍ/UNICRUZ/URI), Regional University of Northwestern Rio Grande Do Sul State (UNIJUI) and Post Graduate Program in Mathematical and Computational Modeling (PPGMMC), UNIJUI, Ijuí, Rio Grande do Sul, Brazil.

[a] 0000-0003-4000-3671
[b] 0000-0003-0205-7775
[c] 0000-0002-1242-5423
[d] 0000-0001-9814-742X
[e] 0000-0003-1907-3341

Published online: 19 January 2024

fuels inflammation but also sabotages the HSR by degrading human antigen R, a crucial mRNA-binding protein responsible for maintaining heat shock transcription factor-1 mRNA expression and stability on heat shock gene promoters. This paper underscores the imperative need to comprehend how chronic inflammation stifles the HSR and the clinical significance of evaluating the HSR using cost-effective and accessible tools. Such understanding is pivotal in the development of innovative strategies aimed at the prevention and treatment of these chronic inflammatory ailments, which continue to take a heavy toll on global health and well-being.

Keywords HSP70 · Heat shock response · Chronic inflammatory diseases · Obesity · Insulin resistance · Exercise

Introduction

Chronic metabolic diseases with inflammatory underpinnings have become prevalent due to the Western lifestyle characterized by high-calorie intake, intestinal dysbiosis, and sedentary behavior. Conditions like obesity, insulin resistance, type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease, cardiovascular diseases (CVD), and neurodegenerative diseases now collectively account for a staggering 74% of global deaths.[1]

Transcriptional profiling studies have unveiled a prominent upregulation of genes associated with inflammatory and stress responses in the adipose tissue of obese individuals.[2] In this Western lifestyle, excessive nutrient intake, coupled with insufficient energy expenditure[3] or skeletal muscle-derived myokines[4–6] to balance cell metabolism and appetite control,[7] adipose cells experience endoplasmic reticulum (ER) stress. Persistent ER stress perpetuates the unfolded protein response (UPR) and triggers the activation of the NOD-like receptor pyrin domain-containing protein-3 (NLRP3) inflammasome in adipose tissue. The ensuing release of inflammatory cytokines leads to a senescence-associated secretory phenotype (SASP), with NLRP3 inflammasome-dependent cytokines "*seeding*" inflammation throughout the body.[3] This chronic ER stress-induced inflammation ultimately culminates in exacerbated insulin release, atherosclerotic vascular diseases, and other chronic inflammatory disorders due to the inability of the organism to resolve inflammation.[3,8] This begs the question: why does a state of low-grade inflammation persist in these metabolic diseases when vertebrates have evolved over millions of years to mount and resolve inflammatory responses? The answer lies in the progressive suppression of the heat shock response (HSR) by proliferative senescence in adipose tissue. The HSR, mediated by heat shock transcription factor-1 (HSF1)-dependent expression of heat shock proteins (HSPs) and other chaperones, is a potent anti-inflammatory pathway that becomes progressively suppressed by proliferative senescence under persistent ER stress and the succeeding NLRP3 inflammasome-driven SASP.[3]

In fact, proliferative senescence of adipose tissue precedes virtually all known forms of chronic degenerative diseases of inflammatory nature, whatever tissue location, from diabetes to CVD, from nonalcoholic fatty liver disease to menopause-associated dysfunctions and neurodegenerative diseases, including Alzheimer's, Huntington's, Parkinson's and most probably fibromyalgia, conditions in which the weakening of the HSR is always observed.[9,10] This story begins by exploring the convoluted link between intracellular chaperone-mediated protein quality control and inflammation. Over the course of billions of years, protein chaperones have evolved with the primary objective of preventing protein misfolding, averting the aggregation of misfolded proteins, and thus thwarting the accumulation of potentially cytotoxic complexes.[11] Actually, although the lexicon "molecular chaperones" is widely recognized in the realm of HSPs, the term was originally coined by Laskey and co-workers[12] to describe these proteins' remarkable ability to prevent incorrect ionic interactions between histones and DNA. Anyway, in the context of a genuine HSR, molecular chaperones play a crucial role in guiding newly synthesized proteins to fold correctly into their functional three-dimensional structures. They effectively prevent aggregation and facilitate the precise localization of these polypeptides through transmembrane translocation. This function holds profound significance in the context of low-grade inflammation, as misfolded proteins or their aggregates exacerbate ER stress (referred to above), thereby triggering inflammatory signals throughout the body, as we shall elaborate upon.

When the HSR experiences irreparable dysfunction, such as when misfolded proteins cannot be salvaged or destroyed, cells have the option to enter apoptosis, effectively halting the generation of inflammatory signals originating from the ER. Conversely, in less severe scenarios where cells do not undergo apoptosis but persistently experience ER stress, they may enter a state of senescence and develop SASP. Therefore, the HSR emerges, on the one hand, as a safeguard of proper functioning within the chaperone machinery that serves to mitigate UPR and, on the other hand, as a potent anti-inflammatory pathway, as we shall elucidate below. Furthermore, the HSR intricately connects protein homeostasis with energy metabolism through interactions between HSF1 and 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK),

an occurrence referred to as "*caloristasis*."[13] Disrupting this delicate equilibrium between proteostasis and energy metabolism can culminate in the formation of aberrant protein aggregates that serve as catalysts for chronic inflammation.

Within the HSP superfamily, the 70 kDa heat shock protein (HSP70) family, hereafter referred to as HSP70, plays a pivotal role in obviating protein misfolding and inflammation.[10,14,15] For the sake of clarity, as outlined in Schroeder *et al.*,[16] HSPs are currently categorized into gene families and superfamilies, aligning more consistently with the HUGO Gene Nomenclature Committee, as indicated by Kampinga *et al.*[17] These include *HSPA* (e.g., HSP70s), *HSPB* (small HSPs), *HSPC* (e.g., HSP90), *HSPD*, and *HSPE* (HSP60 and HSP10 chaperonins), *HSPH* (e.g., HSP110), *DNAJ* (J-proteins, formerly HSP40 cochaperones[18]), alongside *CCT* and other chaperonin-related genes. In relation to *HSP70s*, up to now, the human genome has identified 13 different genes coding for members of the *HSPA*/HSP70 superfamily, such as cytoplasmic/nuclear *HSPA1A*/HSP72 and *HSPA1B*/HSP70-2, ER-based *HSPA5*/GRP78, and mitochondrial *HSPA9*/GRP75. Despite the considerable homology within this superfamily, *HSPA*/HSP70s members exhibit a high degree of specialization. For instance, the response to stress can vary significantly; *HSPA8*/HSC73 (formerly known as the cognate form of HSP70), expressed in both the cytoplasm and nucleus,[19] primarily functions as a non-inducible housekeeping gene. In contrast, *HSPA6*/HSP70B' demonstrates strictly inducible expression, with minimal or no basal expression in most cells. Actually, there is a vast repertoire of combinations of intracellular location and inducibility among HSP members.[20] In this work, however, our primary focus will be on the *HSPA1s*/HSP70s-based anti-inflammatory HSR and its gradual suppression as chronic inflammatory diseases unfold unless stated otherwise.

HSR at the organismal level: Beyond cellular proteostasis to transcellular signaling, intermediate metabolism, and whole-body homeostasis regulation

The HSR serves primarily a protective function *within* cells, but it also evolved to encompass organism-level situations that threaten homeostasis. One such situation is the release of extracellular HSP70 (eHSP70) into the plasma during various stressful conditions (as described below). Indeed, eHSP70 is essentially a composite of both HSP72 (*HSPA1A* and *HSPA1B)* and HSP73 (*HSPA8*) proteins,[21] which may be released in exosomes during stressful situations.[22–26] Unlike intracellular HSP70, which functions as a protein chaperone and anti-inflammatory factor inside the cells, eHSP70 acts as a danger signal and pro-inflammatory cytokine through its interaction with Toll-like 4 receptors outside the cells.[25,27–30] In simple terms, eHSP70 may be produced by a stressed tissue (e.g., during exercise) but eventually acting in others, that is, brain and immune system cells.[25,26] This narrative, however, transcends its current boundaries. As expounded by Schroeder *et al.*,[16] our present understanding of HSF1 activation encompasses four distinct mechanisms, with one of them being cell-nonautonomous (a process initiated not by the stressed cell itself, but rather originating externally, in contrast to the cell-autonomous mechanism) which unfolds within the cell in response to stress signals directed at the cell. This revelation gained prominence following the groundbreaking discovery by Professor Rick Morimoto's research group, shedding light on a previously unforeseen avenue of HSR activation: the transcellular, cell-nonautonomous regulation of HSF1.[31] Accordingly, the nematode *Caenorhabditis elegans* exhibits HSR in a way dependent on thermosensory neurons that detect changes in ambient temperature. As with humans, these specialized neurons exist that play unique roles in controlling temperature-related behavior.[10,32] As a consequence of this relative loss of cellular autonomy in triggering HSF1 activation, the HSR can be attained by the integration of stress-related, metabolic, and behavioral responses of the animal in order to establish a coordinated organismal response to environmental fluctuations.[31]

Transcellular HSR activation is now accepted not to be a peculiarity of *C. elegans*, but a mechanism conserved among metazoans, including mammals.[33,34] This highly elaborated mechanism enables the integration of the HSR across multiple cellular compartments, including the cytoplasm, nucleus, ER, and mitochondria. This is achieved by metabolic adjustment of cell growth, insulin-dependent, and antioxidant factors, which help to compensate for changes in proteostatic load in response to environmental fluctuations.[34] Hence, it became clear that multicellular organisms require the coordination between stress responses and the maintenance of proteostasis, which cannot be achieved through cell-autonomous mechanisms alone. Contrarily, they must be orchestrated in a cell non-autonomous manner through centralized control of the nervous system.[35–38] Remarkably, in *C. elegans*, upregulation of the HSP70 gene (*hspa1a*) through transcellular chaperone signaling (TCS) has been found to increase resilience to heat stress through a unique molecular mechanism. Specifically, TCS is not regulated by the canonical HSF1-dependent HSR, and upon TCS activation, HSF-1-mediated HSR is remarkably suppressed. This favors an *intercellular* route, enabling the animal to preserve its survival. TCS represents an organismal stress response that activates HSP70 expression in an HSF1-independent manner.[38]

While every eukaryotic cell has the necessary machinery for the expression of heat shock genes, the regulation of the

HSR through non-autonomous neuronal signaling to somatic tissues and TCS between neurons and peripheral tissues is a significant evolutionary advancement. This guarantees the coordinated activation of the organismal HSR across various tissues and can supersede neuronal control to restore cell-specific and tissue-specific proteostasis, while maintaining the overall homeostasis, including energy balance. Actually, these mechanisms pungently pinpoint an evolutionarily preserved homeostasis-protecting system at the organismal level that is added to caloristasis,[16] the latter at the cellular level, contributing to the preservation of homeostasis. The understanding of the different metabolic pathways as well as neurotransmitters involved in mammal TCS is still in its infancy. Considering the integrative role of the HSR in responding to diverse intracellular and extracellular stressors, further research endeavors hold the promise of illuminating novel avenues toward a more detailed comprehension of this phenomenon.

In addition to the triggering of the HSR through cell-autonomous and cell-non-autonomous (TCS) mechanisms, there are other well-known mechanisms of HSR activation at the organismal level that attach the autonomic nervous system to the stress responses. The sympathetic nervous system (SNS) branch of the autonomic nervous system serves as the primary conduit for protective responses against disruptions to homeostasis. Remarkably, this role extends not only to vertebrates but also seemingly across the entire metazoan spectrum.[39] As one would anticipate, the evolution of the SNS is intricately linked to the integration of energy sensing mechanisms, encompassing responses to situations ranging from the "*fight-or-flight*" stress response to the regulation of core temperature, glucose homeostasis, and fat storage. Consequently, all the known stimuli that enhance SNS tone indisputably dictate the activation of the anti-inflammatory HSR. Not unexpectedly, SNS plays a proactive role in the mitigation ("*resolution*") of one of the pivotal defensive responses of the animals: inflammation.[40]

The multifaceted interplay of plasma glucose concentrations, energy sensing, HSR, and sympathetic activity takes place within the ventromedial hypothalamus (VMH). This neural hub integrates diverse functions, including feeding regulation, fear response, blood pressure, thermoregulation, and sexual activity. Furthermore, the VMH is the site of a notable estrogen-induced HSR.[41,42] Additionally, mechanisms within the hypothalamus, particularly in the preoptic area, that induce fever (an activator of the HSR) can be modulated by norepinephrine.[32] In the context of hypoglycemia, it is noteworthy that acute episodes trigger the release of eHSP72 and pro-inflammatory cytokines, such as IL-6.[43] Importantly, glucose ingestion effectively inhibits the exercise (stress)-induced secretion of eHSP70[44] and abolishes the counterregulatory actions of the SNS during hypoglycemic episodes.[45] These effects are intricately mingled in the neuronal circuitry within the VMH.[46]

HSP70 expression can be induced by α-adrenergic stimulation in numerous tissues. Conversely, the HSR is impeded by α_1-adrenergic blockade.[47–52] Furthermore, acute stress stimulates the secretion of exosomes containing eHSP70 and augments circulating levels of eHSP70, all in an α-adrenergic-dependent manner. This ultimately serves as a warning to physiological systems of the presence of homeostasis-threatening situations.[22–25] Indeed, the HSR pathway has general protective functions and involves pathways that couple exercise, energy balance, and proteostasis to inflammation *via* HSP70 (Figure 1). To put it differently, HSP70 expression is connected to other homeostatically stressful situations (i.e., homeostasis-threatening situations), *not only heat* or *proteostasis-hostile conditions*.[3,10,25,43] It is worth noting that the perspective depicted in Figure 1 provides insight into just one facet of the HSR. This aspect pertains to situations characterized by *predominantly proteotoxic stress*, where HSF1 takes precedence over AMPK pathways. In contrast, AMPK pathways are favored in scenarios primarily marked by *predominantly metabolic* stress, particularly energy scarcity.

An intriguing facet to contemplate pertains to AMPK, the pivotal cellular energy sensor. In addition to its capacity to discern changes in AMP-to-ATP ratios, AMPK possesses a glycogen-binding domain within its β regulatory subunit.[53] This unique feature endows it with the ability to function as a *glycogen sensor*. In addition, glucose can downregulate AMPK activity through protein phosphatase 2A (PP2A) in various biological contexts, including rodent islets, β-cell strains,[54] and yeast.[55] The activation of PP2A by HSF1 results in the suppression of AMPK,[56] subsequently promoting an increase in HSP70 expression.[57] It is important to acknowledge, however, that, under specific circumstances, AMPK has the potential to *inhibit* HSF1, thereby promoting a pathway focused on safeguarding metabolic functions,[58,59] as proteostasis-saving functions depend on the energy of ATP to provide chaperone-assisted cytoprotection, a phenomenon known as caloristasis,[13] as discussed in detail by Schroeder and colleagues.[16]

Exercise, as it can scarcely be otherwise, resembling a fight-or-flight stressor for the central nervous system (CNS), stands out as a potent inducer of the HSR and HSP70 expression across diverse tissues, *rivaled only by heat*. Notably, exercise prompts the release of eHSP70 into the bloodstream[60,61] and into the culture media of cells derived from exercised subjects.[21,26] This phenomenon is sophisticatedly

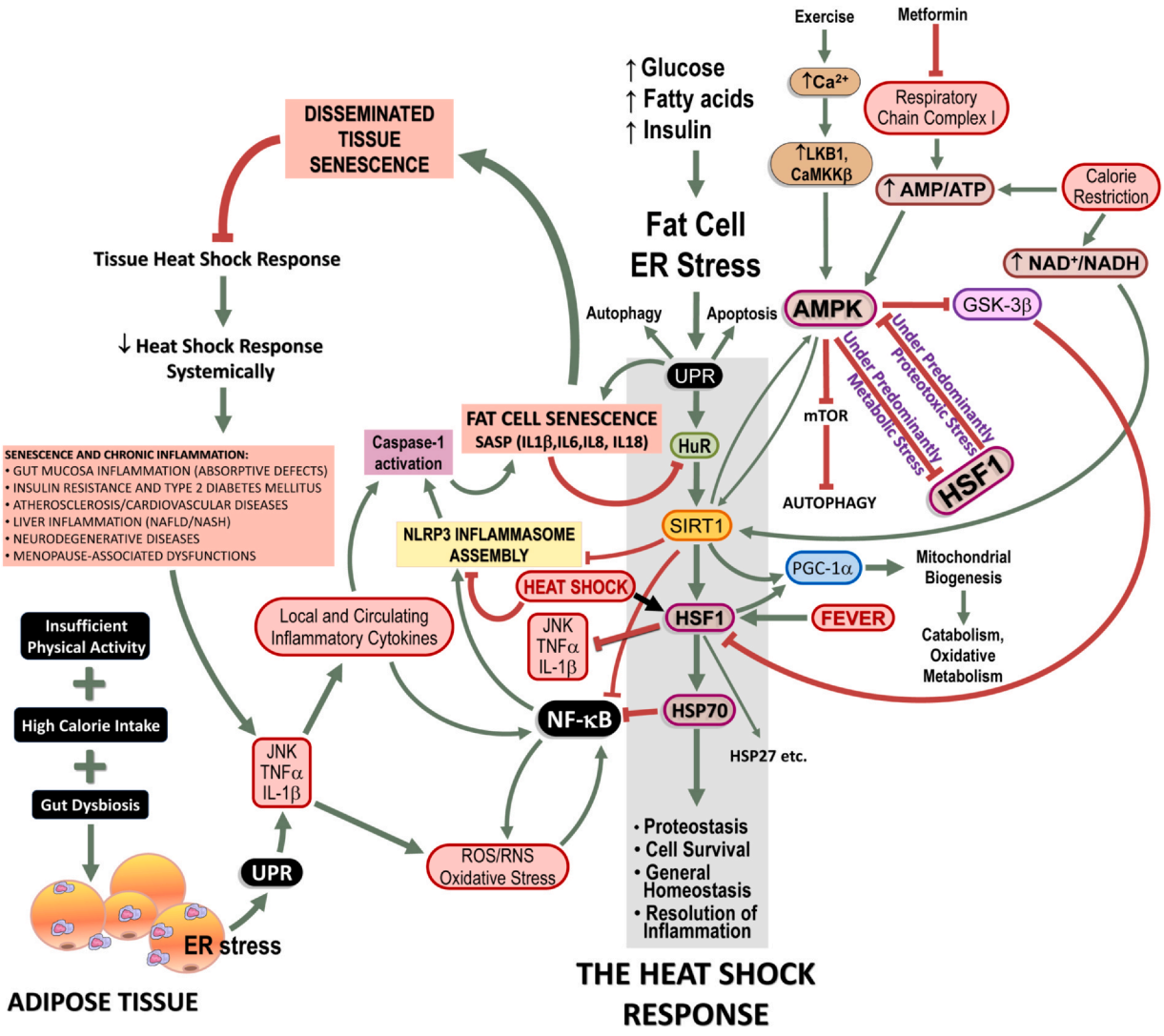

Fig. 1 Heat shock response (HSR) failure in chronic inflammatory diseases: role of cellular senescence. Under normal nutrient supply (i.e., equivalent to energy expenditure, physical activity), glucose and fatty acids are utilized by adipose tissue upon physiological amounts of insulin. Any excess of demand is counteracted by enhanced HSR in order to supply the correct furnishing of chaperones thus avoiding misfolding or correcting endoplasmic reticulum (ER) stress and the resulting unfolded protein response (UPR). When circulating glucose and fatty acids (especially saturated) overcome energy expenditure and high amounts of surplus energetic metabolites should be stored in adipose tissue under a higher insulin command, ER stress develops. Should energy expenditure be still and chronically lower than energy intake, ER stress is followed by the UPR, a cellular strategy evolved in order to evaluate the capacity of the cell to arrange a physiological HSR (which conveys cells to protein/metabolite homeostasis). Of note, the illustration shows essentially one side of the HSR, that involving a predominantly proteotoxic stress in which HSF1 has prevalence over AMPK pathways, which is preferentially activated under predominantly metabolic stress conditions. In the case of irremediable HSR, cells may undergo apoptosis and irreversible cell death. On the other hand, if protein homeostasis (proteostasis) is not attained but cells still have conditions to avoid apoptosis, an alternative metabolic pathway may be taken in which cells do not dye but activate senescence, assuming a senescence-associated secretory phenotype (SASP). This is accomplished because adipocytes chronically challenged by excess fatty acids, cholesterol, high-fat diet, and hyperglycemia prepare an inflammatory response, which becomes chronic. Under the persistence of risk factors, cells develop an UPR that is diverted to the inflammatory branch since continuous inflammatory stimuli do not cease to activate NLRP3 inflammasome, leading to the activation of caspase-1. Activated caspase-1 determines, in adipocytes, a state of frank cellular senescence which culminates in SASP that can spread out to other tissues and cell types, including infiltrating macrophages of adipose tissue, skeletal muscle cells, pancreatic β cells, hepatocytes, vascular cells, and brain structures. In all these cell types, including adipocytes, SASP leads to cleavage of HuR, an mRNA-binding protein responsible for enhancing SIRT1 expression. As a consequence, HSF1 expression and transcribing activity becomes depressed, because SIRT1 enhances both. Therefore, HSR is hindered accordingly and a state of enhanced inflammation is noted because HSR is of crucial importance for the resolution of inflammation for many reasons, including, but not limited to, HSF1-dependent blockade of pro-inflammatory cytokine expression and impairment of NF-κB activation. As a healthy HSR cannot resume, resolution of inflammation is more and more jeopardized thus impeding autophagy and an efficient resolution of UPR *via* HSR. Beside of this, senescent cells are resistant to undergo apoptosis, which should be an alternative to break this vicious cycle, so that chronically

inflamed cells are likely to persist in tissues. Abbreviations used: AMP, adenosine monophosphate; AMPK, 5'-adenosine monophosphate-activated protein kinase; HSF1, heat shock transcription factor-1; HSP70, 70 kDa family of heat shock proteins; HuR, human antigen R; JNK, c-Jun N-terminal kinase; NF-κB, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; NLRP3, NOD-like receptor pyrin domain-containing protein-3; ROS, reactive oxygen species; SIRT1, NAD^+-dependent protein deacetylase of class III family sirtuin-1; TNF-α, tumor necrosis factor-α. *Adapted and reused from*: Ref. 9. Under an open access Creative Common CC BY license 4.0.

linked to heightened immunosurveillance,[62,63] a process known to be orchestrated *via* α_1-adrenergic signaling.[23] Conversely, the consumption of glucose during exercise negates the expected surge in circulating eHSP70 levels.[44] Notably, the expression of HSP70 and its release into the bloodstream are closely associated with indicators of cellular energy depletion. Accordingly, diminished glycogen levels are directly correlated with elevated expression, both at the mRNA and protein levels, of HSP72.[64] Furthermore, this upregulation of HSP72 mRNA levels correlates with the onset of exhaustion, a stimulus integrated at the SNS level,[65] while displaying an inverse relationship with lactate levels. Noteworthily, muscle temperature elevation primarily occurs at the outset of the exercise session.[66]

Following an acute bout of exercise, an increase in HSP70 and HSP90 levels is observed in plasma, mononuclear cells, and various organs and tissues, including muscle, liver, cardiac tissue, and brain. This specific response of HSP70 and HSP90 appears to be closely related to the exercise modality, with several dependent factors, such as exercise duration, intensity, type, the training status of the subjects, and environmental factors, such as temperature.[67] Actually, as is the case of HSP70, HSP90 is also now considered a potential myokine (or exerkine[68]) and mediator of exercise-induced immune responses in patients with idiopathic inflammatory myopathies.[69] Interestingly, after repeated bouts of eccentric exercise, HSP70 and small HSPs accumulate in areas of myofibrillar disruption and sarcomeres, particularly in Z-disks.[70]

Still, regarding the connection between the HSR and the metabolic stress surveillance system, it is worth noting that, under predominantly proteotoxic stress conditions (Figure 1), AMPK plays a pivotal role in triggering the HSR by inhibiting glycogen synthase kinase-3β (GSK-3β), a protein that constitutively represses the activity of HSF1. GSK-3β serves as a negative regulator of glycogen synthase and is typically deactivated when the body is in an "*energy plentiful*" state, thereby liberating the conversion of glucose into glycogen. In contrast, AMPK serves as the primary "*energy-sensing*" kinase that responds to periods of "*fuel scarcity*" stress.[3,10,71] It is also important to acknowledge that the impact of AMPK on the HSR is contingent on the prevailing caloristasis, meaning that during predominantly metabolic stress conditions, AMPK suppresses HSF1 activity, resulting in a dampened HSR. Conversely, under conditions characterized by predominantly proteotoxic stress, HSF1 can counteract the effects of AMPK, leading to a *bona fide* HSR.[16]

The AMPK-mediated alleviation of GSK-3β's inhibitory influence on the HSR may occur through the hexosamine biosynthetic pathway (HBP), a nutrient-sensing pathway with extensive ties to energy metabolism and the HSR, beyond its role in glycogen synthesis.[9,72,73] Glutamine, the primary amino acid released into the bloodstream by active skeletal muscle,[9,71,74,75] and a significant co-inducer of HSP70, enhances the HSR by increasing the HBP metabolic pathway which, in turn, leads to the inhibition of GSK-3β. Activated AMPK, in this situation, relieves the inhibition of HSF1, ultimately contributing to an anti-inflammatory HSR.[9,76] Notably, in the context of protein-aggregation disorders like Huntington's disease, phosphorylation of Ser303 and Ser307 by GSK-3β results in the inactivation and degradation of HSF1.[77]

Turning our attention to the potential impact of exercise on homeostasis again, it becomes evident that exercise exerts a mechanical force, essentially a form of traction, on the skeletal structure. Given this, it was not unreasonable to anticipate that the physiological stress induced by exercise might extend to the bones. Intriguingly, this is rigorously the case. When bony vertebrates encounter immediate danger, stress signals in the basolateral amygdala of the brain trigger the release of glutamate by glutamatergic axons that contact osteoblasts in the bone. This triggers the secretion of osteocalcin, a bone-derived hormone (an osteokine[78]) that actively contributes to energy metabolism, reproductive processes, memory, and the capacity for exercise. These multifaceted functions are indispensable for thriving in unpredictable and hostile environments, such as the wilderness.[79] Additionally and remarkably, osteocalcin plays a critical role in suppressing the tonus of the parasympathetic nervous system, which allows the stress response to occur because the sympathetic tone remains unopposed. As a result, osteocalcin is released from the osteoblasts as part of the acute stress response, which turns off the "*rest-and-digest*" arm of the autonomic nervous system and enables the acute stress response to proceed *via* the "*fight-or-flight*" sympathetic branch.[79] Osteocalcin-mediated stress response results in a range of physiological manifestations, including increased body

temperature and energy expenditure, elevated heart rate, and faster breathing.[79] Bone-derived osteocalcin favors catecholamine synthesis in the brain, thus enhancing sympathetic tone which is the principal efference of the acute stress response[80] and a major positive signal to trigger the HSR.

In concluding this discussion linking the stress responses among the SNS, metabolism, and exercise, it is crucial to highlight the pivotal role of the skeletal system as an endocrine organ in regulating glucose and energy metabolism. The interplay between bone and energy metabolism has long been a subject of interest, fueled by the observation of an inverse relationship between obesity and osteoporosis.[81] Bone-derived osteocalcin stimulates insulin sensitivity, insulin secretion, and energy expenditure by upregulating the expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), uncoupling protein-1, and mitochondrial biogenesis. Therefore, it is a typical homeostasis-protecting hormone. Also, its secretion and activity are modulated by various hormonal cues, including insulin, leptin, SNS activity, and glucocorticoids.[82] Essentially, the effects of osteocalcin converge on the same metabolic pathways as those associated with the HSR. Furthermore, it is remarkable that serum osteocalcin levels are notably lower in obese children compared to their normal-weight counterparts, underscoring a negative correlation between osteocalcin levels and body fat mass.[81]

To summarize organismal HSR at this point, all known physiological responses to any kind of stress are definitely connected to the HSR. As inferred from the above findings, it is clear that the acute stress response of the skeleton involves metabolic signals similar to those managed in the HSR. Curiously, these pathways are also activated by osteocalcin under emotional stress (e.g., fear). They are the same metabolic routes activated under metabolic "*fuel shortage*" stress that triggers the expression and export of HSP70 to the extracellular milieu (eHSP70), as detailed above. Conversely, different HSPs influence the behavior of osteoblasts, osteoclasts, and osteocytes in the bone, thus participating in bone physiology. For example, although still a matter of debate, the chaperonin HSP60 (*HSPD*) stimulates osteoclast activity leading to bone resorption, as do HSP73 (*HSPA8*) and HSP90 (*HSPCs*).[83] On the other hand, treatment of osteoblasts with HSR inducers, such as sodium arsenite or heat stress, led to the attenuation of osteocalcin synthesis induced by either bone morphogenetic protein-4 or the thyroid hormone T_3, along with an induction of HSP27 (*HSPB1*). Actually, current evidence strongly suggests that unphosphorylated *HSPB1*/HSP27 exerts an inhibitory effect on osteocalcin synthesis by a stimulatory effect on mineralization in osteoblasts.[84] *HSPB1*/HSP27 is a molecular chaperone that operates independently of ATP and has been linked to tumorigenesis, metastasis, and protection against heat stress.[85] Its function is influenced by its dynamic phosphorylation and heterogeneous oligomerization under varying conditions of stress. When unphosphorylated, HSP27 (*HSPB1*) can form large multimers of up to 800 kDa. However, phosphorylation triggers conformational changes resulting in significantly smaller oligomeric sizes, complex dissociation, and loss of chaperone activity. This indicates that HSP27 (*HSPB1*)'s reversible structural organization ultimately functions as a sensor that enables cells to adapt and overcome lethal conditions by interacting with appropriate protein partners.[86] Therefore, it seems plausible that HSP27 (*HSPB1*) liberated by the skeletal muscle after exercise may be involved not only in tissue protein repair but also in disarming SNS after exercise. This signifies that evolution may have picked out this mechanism to say "danger has passed" and there is no longer a need to activate the *fight-or-flight* response.

In total, the HSR pertains to a general surveillance system that connects cellular proteostasis to physiological adjustments in blood pressure, cardiac output, temperature, glycogenolysis/glycogen synthesis (to correct glycemia), capacity to exercise (fight-or-flight), and affective (limbic) brain responses to *already-experienced* and *unpredicted stresses*, thereby *anticipating* such responses.

The HSR and the physiological resolution of inflammation

As stated in the Introduction section, there is a close association between intracellular quality control of proteins and inflammation. But what is the relation between protein aggregation and unresolved inflammation that permits the existence of chronic metabolic diseases of an inflammatory nature? The answer is that, because of many evolutionary reasons, activation of the HSR, does exert anti-inflammatory effects in animals, and, as we shall discuss here, HSR is progressively suppressed during the establishment of these chronic metabolic diseases, making inflammation perpetual.

Prior to acknowledging the significance of the HSR in the context of inflammation and exploring its role in the resolution of inflammation, it is essential to clarify a crucial point. When there is an excessive demand placed on ER functions, specifically concerning protein synthesis and proper folding, a cellular process known as the

UPR is activated to prevent the accumulation of potentially cytotoxic misfolded protein aggregates. However, within the UPR framework, one of its branches, namely *I*nositol *R*equiring *E*nzyme-1 (IRE1)/c-Jun *N*-terminal kinase-1 (JNK1) (discussed next), can physiologically incite an inflammatory response that depends on the nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family (NF-κB) downstream pathways, ultimately triggering inflammation. Conversely, this same process also induces the expression of molecular chaperones. Consequently, the HSR emerges as a vital mechanism to counteract any excessive inflammatory response triggered by a potent UPR. This elucidates a significant aspect of the HSR in resolving inflammation. In essence, the HSR evolved as a potent anti-inflammatory tool, strategically harnessed to combat various forms of inflammation.

The HSR exerts cogent anti-inflammatory effects, primarily because HSP70s (*HSPAs*) act as natural inhibitors of NF-κB subsequent signaling cascades. NF-κB activation is sufficient and *sine qua non* to trigger inflammation since all known inflammatory cytokines and mechanisms involved in inflammation are NF-κB-dependent. HSP70s stabilize the inhibitors of κB (IκB) complexes with NF-κB, thus preventing the translocation of the active dimers (e.g., p50/p65) into the nucleus and the consequent transcribing activity.[87,88] Many other forms of HSP70-dependent anti-inflammatory activities have also been reported.[89] They include induction of HSP70-dependent anti-inflammatory cytokines, modulation of dendritic cell phenotype favoring Th_2-over-Th_1 lymphocyte differentiation, and preferential activation of T_{reg} cells.[89]

Inflammation is a well-preserved response intended to eliminate microorganisms or prepare tissue recovery during an aseptic injury. In a nutshell, inflammation evolved as an adaptive response for restoring homeostasis.[90] Importantly, immune responses related to inflammation can be appreciated in all metazoans.[91] These responses are usually acute, in that cells of the innate immune system are recruited to the site of sterile injury or to defeat microorganism invasions. Having achieved this objective, inflammation is rapidly dismantled. A successful acute inflammatory response culminates in the eradication of infectious agents (or the start of tissue remodeling), followed by a *phase of resolution* and tissue repair.[90] That is to say, chronic inflammation was not part of the original evolutionary blueprint; much less chronic inflammatory diseases.

When an acute inflammatory response is activated in the body, a significant production of pro-inflammatory arachidonic acid-derived prostaglandins (PGs) occurs, along with other lipid mediators and vasoactive compounds.[10] These signaling molecules play a decisive role in alerting immune cells and sensory pathways to the presence of an invader or tissue injury. They also increase vascular permeability, which allows more inflammatory cells to arrive and activate, leading to tissue repair.[90] This process is initiated by a finely orchestrated expression of inducible proteins centered around NF-κB transcription factors, which drive inflammation during the initial phase.[92] However, these same molecules also contribute to the *resolution of inflammation*.[3,10,93–95] Additional polyunsaturated fatty acid-derived mediators, such as lipoxins and dietary ω3-fatty-acid-derived resolvins and protectins, further facilitate the resolution of inflammation and tissue repair.[90]

Within 2 h of the start of an inflammatory response, a powerful wave of NF-κB-induced expression of cyclooxygenase-2 (COX2, formally known as PGs endoperoxide H synthase) surges through the body tissues, synthesizing copious amounts of PGE_2 from arachidonic acid. This early phase can be inhibited by both selective COX2 inhibitors (COXIBs) and traditional dual COX1/COX2 nonsteroidal anti-inflammatory drugs. However, there is an unpredicted and serious risk here: using such inhibitors to alleviate fever, headache, or body pain, at any time during an inflammatory response, strongly exacerbates inflammation at later stages (after 48 h from the beginning), impeding its resolution phase and prolonging or perpetuating the inflammatory state.[94] This occurs due to various causes. Firstly, PGE_2 is a potent inducer of fever, by impeding the processing of thermosensory information at the preoptic area of the hypothalamus.[32,96,97] This PGE_2 signal then triggers autonomic heat-sparing mechanisms located at the rostral medullary raphe pallidus premotor nuclei, setting off a cascade of bodily responses, including cutaneous vasoconstriction, the feeling of cold and, prominently, elevation of core temperature (fever). Therefore, the initial phase of inflammation (PGE_2-driven) tends to induce *fever* (*the most potent physiological HSR inducer*) precisely to prepare the termination phase (resolution) so that COXIBs or conventional nonsteroidal anti-inflammatory drugs gravely jeopardize the physiological resolution of inflammation. Strikingly, fever effectively orchestrates antimicrobial defenses and aids in controlling inflammation and tissue repair, even in cold-blooded vertebrates (!), where there is selectivity in the immune mechanisms activated by fever. In summary, fever inhibits inflammation and significantly enhances wound repair[98] throughout the metazoan kingdom. Because the HSR is a multifaceted anti-inflammatory and antiviral mechanism that naturally resolves inflammation, selective manipulation of this pathway has the potential to control and prevent multifactorial diseases.[99] Quite the contrary, however, long-term

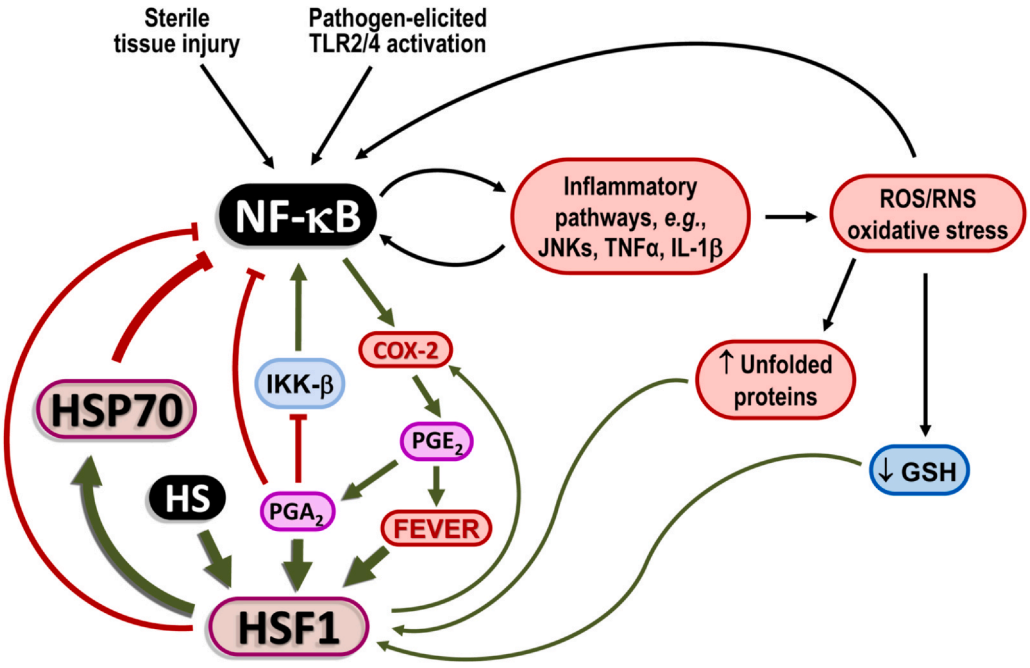

Fig. 2 Heat shock response (HSR) dynamics in inflammatory responses. Highlighted is the dual function of NF-κB in promoting pro-inflammatory cytokine production and priming the resolution phase *via* COX-2 activation. Abbreviations used: COX2, cyclooxygenase-2; HSF1, heat shock transcription factor-1; HSP70, 70 kDa family of heat shock proteins; IKK-β, IκB kinase-β JNK, c-Jun N-terminal kinase; NF-κB, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α

use of COXIBs obviates the physiological triggering of the HSR and, as a result, has been shown to be harmful to human health.[100,101] This harm has led to the withdrawal of rofecoxib and valdecoxib from the market due to their association with cardiovascular complications.[101] Let us not forget: the HSR is also anti-inflammatory in the cardiovascular system. The second point regarding the negative impact of blocking COX activity is that PGE_2 induces fever in the first step but, shortly after, it undergoes dehydration in the plasma into the highly electrophilic cyclopentenone PG (cyPG) PGA_2,[102] which is a powerful antiproliferative eicosanoid that can shut off NF-κB directly and also *via* HSF1 activation.[103–105] In addition, heat induces HSF1-dependent COX2 expression, leading to explosive PG production and HSP70 synthesis, which inhibits NF-κB-dependent pro-inflammatory cascades.[106] Please, see Figure 2 for the detailed involvement of COX-2 in the HSR.

Anti-inflammatory A-type PGs may also exert an antiproliferative effect by operating akin to statins, as they bind directly and covalently to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase.[107] This enzyme serves as the rate-limiting step in cholesterol synthesis. However, the inhibition of HMG-CoA reductase not only prevents the formation of cholesterol, the final product of this pathway but also hinders cell proliferation. This is because various isoprenoid intermediates, essential for cell proliferation, are synthesized between HMG-CoA and cholesterol. In essence, disrupting cholesterogenesis leads to the inhibition of cell proliferation.[108,109] Strikingly, inducers of the HSR, particularly anti-inflammatory cyPGs, at the same time, potently inhibit viral replication in various DNA and RNA viruses, including HIV-1, *via* HSF1-dependent inhibition of NF-κB activation and HSR activation.[103,110–117] Blocking virus replication by cyPGs depends on HSP70 synthesis, which explains why hyperthermic treatment is antiviral, including in the fever-like range.[118] Beyond the intracellular effects against inflammatory pathways already discussed, the HSP70 family of chaperones is also involved in the response to viral infections. HSP70 plays an important role during virus internalization, replication, and gene expression.[119]

During the most important virus-elicited pandemic of this century, many research groups suggested a possible link between the major susceptibility to infection with severe acute respiratory syndrome coronavirus-2, in patients with metabolic diseases presenting chronic failure to trigger a robust HSR.[120–123] Administration of recombinant HSP70 *in vitro* and *in vivo* has been found to decrease inflammation and respiratory distress syndrome in severe acute respiratory syndrome coronavirus-2 infection.[124] At the moment, only one study by our own team has assessed the HSR in COVID-19 patients. However, we did not find a difference in the HSR between critical COVID-19 patients with and

without T2DM, because of a worsening of HSR in both groups, when these patients' results were compared with normal never-infected individuals.[125] HSR impairment in severe COVID-19 patients appears to be independent of previous metabolic status. However, we assessed only severe COVID-19 patients. Hence, further research is required to discern whether the severity of COVID-19 itself dampens the HSR and/or whether individuals with a robust HSR prior to infection are less likely to experience worsening outcomes.

One of the evolutionarily oldest mechanisms involved in the termination of inflammation is certainly the HSR. This is not a mere coincidence. During an inflammatory response, ER is highly demanded to give rise to and prepare immunoglobulins and pro-inflammatory cytokines in immune cells. This must be closely supervised by HSP chaperones. If a minimal imbalance between protein synthesis and chaperoning activity is perceived by ER molecular sensors, it is imperative for the cell to halt protein synthesis and increase its chaperoning capacity immediately to avoid the accumulation of potentially toxic protein aggregates. If this occurs, aggregates of unfolded proteins are conveyed to HSP70/*HSPA1s* (or HSP90/*HSPCs*) to be refolded or conduced to proteasome-dependent degradation in the case of irrecoverable misfolding. On the other hand, if ER is overburdened with excess unfolded proteins, UPR takes place in order to suspend protein synthesis and enhance the production of HSP70s (and other chaperones). Notwithstanding, UPR has an inflammatory branch that can be counteracted by the concomitant production of the anti-inflammatory HSP70 under physiological conditions. This closes the feedback loop between inflammation and the physiological resolution of inflammation through the control of proteostasis while opening a window to the understanding of the suppression of the HSR in chronic inflammatory diseases.

Impaired resolution of inflammation due to progressive suppression of HSR in chronic-degenerative inflammatory diseases

The connection between chronic inflammation and the HSR involves a very complex network that operates at the gene regulatory level. The tumor necrosis factor-α (TNFα) gene promoter, for example, contains an HSF1 binding site that represses TNFα transcription.[126] Consequently, knockout of HSF1 gene is associated with a chronic augmentation of TNFα levels, increased susceptibility to endotoxin challenge,[127,128] and NF-κB/AP-1-mediated exacerbation of angiotensin-II-induced inflammation in vascular smooth muscle cells.[129] On the flip side, TNFα can temporarily inhibit HSF1 activation.[130] Additionally, the pro-inflammatory kinase, JNK1, phosphorylates HSF1 in its regulatory domain, leading to the suppression of HSF1 transcriptional activity. On the other hand, HSP70 may inhibit the JNK pathway to prevent apoptosis.[128,131] Exposure of cells to heat shock and other protein-damaging conditions, including ethanol, arsenite, and oxidative stress, strongly decreases the rate of JNK dephosphorylation,[132,133] thus inhibiting JNK activity.

HSF1 suppresses IL-1β gene transcription by forming a complex with the nuclear transcription factor of interleukin-6 (NF-IL6), which directly inhibits IL-1β gene promoter activity.[134] However, NF-IL6 can prevent HSF1 activation by blocking its transcriptional activity and displacing HSF1 from the heat shock elements in the promoters of heat shock genes.[135] Additionally, IL-6 can depress HSF1 transcribing activity *via* GSK-3β activity.[136] HSF1 and NF-IL6 have mutually antagonistic effects,[137] and the preponderant effect depends on the most prevalent stimulus, as observed for several other pro-inflammatory genes.[138] These interactions depend also on the degree of predominance of *metabolic* or *proteotoxic stress* (*caloristasis*), as approached in the previous sections. Therefore, a delicate balance is required to equilibrate pro-inflammatory and anti-inflammatory responses depending on the overall signals that converge to HSF1 and its regulators. In T2DM, the disruption of insulin signaling leads to HSF1 deactivation, as GSK-3β constitutively inhibits HSF1 through direct phosphorylation. This reduces HSR activity and subsequently fosters heightened activation of pro-inflammatory cytokines, JNK, and IκB kinase (IKK)-β. These signals, in turn, lead to the phosphorylation of Ser307 of insulin receptor substrate 1 (IRS1), further impeding insulin signaling.[139]

Since T2DM and its complications have oxidative stress as an underlying mechanism, and considering that HSPs are major protective molecules against oxidative stress, Kurucz and colleagues tested and demonstrated, for the first time, the hypothesis that HSP72 (*HSPA1s*) mRNA contents should be undermined in the skeletal muscle of T2DM patients. In fact, they observed that decreased levels of HSP72 mRNA in the skeletal muscle of T2DM patients were correlated with a decreased rate of glucose uptake by cells and insulin resistance.[140] In T2DM and obesity, decreased expression of both HSP70 and HSF1 is a common feature detected in adipose tissue, liver, skeletal muscle, and vascular beds of patients.[30,140–149] Depressed HSR is also evident in menopause-related metabolic dysfunctions[10,150] and in tissues of obese[151] and older adults,[152–154] including those presenting neurodegenerative diseases.[9,155] Impaired HSR has also been reported in rodent models of obesity,

insulin resistance, and CVD.[153,156–158] Of note, pharmacological[159] as well as non-pharmacological interventions[156,160] intended to activate HSF1 have been evaluated to block or reverse such unhealthy conditions *via* enhancing HSR successfully. Additionally, protein aggregation-related neurodegenerative diseases are also directly linked to a deficiency in the expression and function of HSF1, particularly to its degradation and loss of DNA binding activity.[77] HSP70s, on the other hand, provide protection against an array of brain disorders, including trauma and stroke,[161,162] not necessarily linked to protein aggregation directly.

As we discussed in the previous sections, ER stress can be resolved in various ways, such as increasing the capacity of protein chaperoning and decreasing protein synthesis. When the demand for ER functions exceeds its ability to cope with protein synthesis without accumulating misfolded proteins, the ER proteostasis surveillance system, under ER stress, triggers the UPR (to avoid undesirable tautology, please refer to the excellent review by Hetz *et al.*[163]). UPR, in turn, has two major biochemical pathways to resolve ER stress, the *adaptive* and the *proapoptotic* routes. The first was evolutionarily selected to protect cells from proteotoxic oxidative stress while increasing protein chaperoning and refolding. The second is activated when the capacity of UPR to sustain proteostasis is exceeded and cells enter apoptotic programs. Within the adaptive UPR, there are three known main routes responsible for the re-establishment of proteostasis without the need for condemnation of cells to die by apoptosis: the *P*rotein kinase-like *ER K*inase (PERK)-dependent pathway, the *A*ctivating *T*ranscription *F*actor 6 (ATF6)-dependent pathway, and the IRE1α also known as ER-to-Nucleus Signaling-1)-dependent pathway. PERK route is in charge of translation attenuation to reduce protein folding load, selective translation, autophagy, and antioxidant responses. The ATF6 pathway regulates the transcription of genes that encode ER chaperones, enzymes that facilitate ER protein translocation, folding, maturation, and secretion, as well as proteins involved in the degradation (ER-associated protein degradation) of misfolded proteins. ATF6 functions transit between ER and Golgi apparatus. Finally, IRE1α-dependent pathways regulate protein loading in the ER, metabolic adaptation, autophagy, ER-associated protein degradation, and NF-κB-dependent inflammatory signals *via* the NLRP3 inflammasome. Furthermore, IRE1α can associate with adapter proteins to engage in crosstalk with other stress response pathways such as macroautophagy and the MAPK pathways.[163]

Hence, evolution selected a protein-folding correction system to resolve UPR with a *momentary inflammatory profile*, at the same time the HSR works as a sentinel to supervise both protein chaperoning activity and NF-κB-dependent signals to avoid unwanted inflammation from being triggered during UPR. For these reasons, when examining the low-grade inflammatory background that accompanies *all* chronic degenerative diseases of an inflammatory nature,[3,159,164] the question arises: why does the HSR fail to eliminate UPR-elicited inflammatory signals caused by excessive ER stress? Why does the HSR not function to promote the physiological resolution of inflammation in these cases? The answer to these questions lies in the fact that metabolic stresses resulting from energy imbalance (surplus) and insufficient physical activity continually require providence from the ER. This leads to the sustained maintenance of ER stress and exacerbated UPR. Additionally, both the adaptive and proapoptotic UPR pathways ultimately have inflammatory branches that rely on the activation of NLR-family inflammasomes (including NLRP1, NLRP3, NLRP6, and NLRC4), which promote the conversion of procaspase-1 to *c*ysteine-*asp*artate prote*ase*-1 (caspase-1) (formerly known as interleukin-1 converting enzyme).

Inflammasomes are evolutionarily marvelous complexes of molecular platforms that sense and respond to danger signals. These large multimeric complexes are responsible for promoting the activation of caspase-1, a crucial enzyme involved in immune and inflammatory responses. In addition to activating caspase-1, inflammasomes also play a key role in cleaving inactive pro-interleukins and other target proteins, ultimately leading to the production of their active forms. Although all NLR-type inflammasomes can activate caspase-1, the NLRP3 inflammasome has been studied the most extensively.[165,166] Importantly, NLRP3 inflammasomes have proven to be involved in the noxious effects of permanent ER stress, as we shall discuss next.

Under chronic ER stress, PERK-elF2α-ATF4-CHOP and IRE1-JNK pathways are activated from the lumen of ER to prepare the UPR or initiate apoptosis in mammalian cells.[167] In particular, IRE1, which is a serine/threonine-protein kinase/endoribonuclease, connects ER stress to NF-κB-dependent inflammatory signals. Accordingly, in response to ER stress, maximum activation of NF-κB requires the presence of the IKK to address the IκB to proteasome degradation. However, unlike canonical activation of NF-κB, the activity of IKK does not increase during ER stress. Instead, the extent of NF-κB activation depends on the level of *basal IKK activity*, which is crucial for determining the response. In this way, IRE1, a critical initiator of the UPR, contributes to maintaining the basal activity of IKK through its kinase activity but not its RNAse activity.[168] As a consequence of the above ER mechanisms, if the flow through the UPR overcomes the cellular ability to repair proteins, the inflammatory branch of the UPR overwhelms its anti-inflammatory capacity (*via* HSP70s) and a huge burst of

NF-κB-dependent pro-inflammatory cytokines begins. This is exactly what happens in adipose tissue when energy intake surpasses energy expenditure, as stated in the Introduction section. And more: an endless ER stress accompanied by strong UPR may occur in adipose tissue even without any apparent obesity. In other words, overburdened UPR and low-grade inflammation may ensue in apparently lean individuals, as is the case of lean T2DM patients.[169] Indeed, the major point here is the "*metabolic stress*" to the adipose tissue (i.e., adipocytes and their satellite cells, such as macrophages), not overweight or obesity itself. Unfortunately, however, chronic degenerative diseases of an inflammatory nature are all associated with progressive UPR-dependent suppression of the HSR.

When ER stress in adipose tissue remains constantly utilizing the pro-inflammatory pathways of the UPR, the permanent activation of the NLRP3 inflammasome is inevitable. However, in this situation, various pro-inflammatory cytokines are activated (after cleavage of their respective pro-cytokines by caspase-1). This results in the incessant production of caspase-1, which in turn allows for the activation of large quantities of pro-inflammatory cytokines.[170,171] These cytokines affect adipose tissue but are also released by it into its surroundings.[3] Consequently, "aberrant" activation of the NLRP3 inflammasome is implicated in various inflammatory disorders, such as cryopyrin-associated periodic syndromes, Alzheimer's disease, diabetes, and atherosclerosis that occur far from adipose tissue.[3,165,172–174] The persistent activation of the NLRP3 inflammasome has another facet: the establishment of a pattern of pro-inflammatory cytokine production known as the SASP, which was mentioned in the Introduction section. As shown in Figure 1, fat cell senescence and the associated SASP may be an alternative mechanism emanating from the UPR that cells employ *to avoid apoptotic death* when autophagy is not working and the anti-inflammatory HSR fails to operate.[3] In fact, a senescent-like state can emerge in the fat cells of obese individuals (even young obese subjects), as an adaptation to the overutilization of such cells, which resembles cellular aging.[175] Additionally, high-fat diet (HFD)-induced obesity can lead to vascular senescence through long-term activation of Akt1 and mTOR.[176] As proliferative senescence serves as an alternative to apoptosis, it would represent an interesting evolutionary solution that could *preserve dysfunctional cells*, although *at the expense of the organism*. Teleologically, this may seem counterintuitive as it undermines the overall health of the organism. On the other hand, it appears clear that evolution did not anticipate the extent to which humans would challenge the biochemistry originally "designed" for the intermediary metabolism of an animal consuming a Paleolithic diet (consisting of lean meat, fruits, vegetables, and nuts, but not grains, dairy, or legumes) and engaging in hunter-gatherer levels of physical activity.[3]

Cellular senescence, or proliferative senescence, is a part of the cellular stress response. Senescent cells are identified by a combination of features and molecular markers, including essentially permanent growth arrest. However, no single characteristic is exclusive to the senescent state and not all senescent cells display all known markers. Therefore, senescent cells are typically identified by a conjunct of characteristics,[177] which will not be approached here.

Now, we present evidence revealing how the HSR is profoundly obstructed by cellular senescence in response to the abundant activation of UPR. Human fibroblasts from adult segmental progeroid Werner syndrome undergo premature senescence that is associated with a strong positive feedback system in which overactivation of the p38-NF-κB pathway in these cells leads to SASP, which then attenuates the expression of the mRNA-binding protein human antigen R (*HuR*; also known as embryonic lethal, abnormal vision, Drosophila, homolog-like protein-1). HuR is a critical factor for the activity of the NAD^+-dependent protein deacetylase of class III family sirtuin-1 (SIRT1).[178,179] Indeed, the copious amount of NLRP3 inflammasome-originated caspase-1 progressively destroys the available quantities of HuR because caspase degrades HuR.[180] Caspases can also mediate the cleavage of HuR under different situations.[181] Of note, HuR enhances the stability of various target mRNAs, such as the one encoding SIRT1. This occurs through the association of HuR with the 3′-untranslated region of SIRT1 mRNA, leading to an increase in SIRT1 protein expression levels.[178] However, oxidative stress, which is a potent activator of NLRP3,[170] disrupts the interaction between HuR and SIRT1 mRNA. This leads to a decrease in cell survival through a cycle checkpoint kinase-2-dependent mechanism.[178] SIRT1, on the other hand, enhances the expression of HSF1[179] and, when activated, SIRT1 prolongs HSF1 binding to the promoters of heat shock genes by maintaining HSF1 in a deacetylated, DNA-binding competent state.[126,182] Furthermore, heat shock itself increases the cellular NAD^+/NADH ratio and enhances the recruitment of SIRT1 to the HSP70 promoters.[183] Conversely, SIRT1 knockdown attenuates the HSR,[184] while SIRT1 modulators were found to also modulate HSF1 activity and the HSR in human cells.[183]

During caspase-mediated apoptosis, HuR undergoes a functional switch from pro-survival to pro-apoptotic.[185] Interestingly, attenuation of HuR sensitizes adenocarcinoma cells to apoptosis.[186] However, in the

context of continuous NLRP3 inflammasome activation, the ability of HuR to induce apoptosis[187] is inhibited. This is due to the caspase-dependent degradation of HuR, which triggers a process of senescence-associated SASP, ultimately preventing the cells from undergoing apoptosis. This is a regrettable circumstance since apoptosis of cells relaying inflammatory signals would have the potential to relieve the organism of the SASP that induces inflammation everywhere.

After a cellular insult, such as genotoxic stress, HuR binds to SIRT1 mRNA, triggering an anti-apoptotic and pro-survival gene expression program.[188] However, the involvement of HuR in cellular homeostasis extends beyond this function. It plays a role in the differentiation of pre-adipocytes by regulating the translation and stability of GLUT1 mRNA, indicating its importance in muscle and adipose tissue differentiation processes.[189] Reduced HuR levels are associated with increased cellular senescence. This is why HuR is considered the patron of the "young cell" phenotype.[190] In addition, HuR works in synergy with heat shock and calorie restriction, which enhance SIRT1 deacetylase activity, to respond to heat shock.[183]

The tangled interplay between HuR-dependent HSR and the metabolic state of cells extends further. On the one hand, HuR regulates SIRT1 mRNA expression and stability, consequently influencing HSF1 expression and activity. On the other hand, HSF1 may fine-tune the transcription of HuR in a complex interplay involving AMPK, HuR, and SIRT1.[59] Despite HuR may autoregulate its own expression through alternative polyadenylation site usage,[191] HSF1 exerts tight control over HuR transcription.[59,192–195] For HuR to operate adequately as an mRNA-binding protein, it has to be exported from the nucleus to the cytoplasm. Nevertheless, in *predominantly metabolic stress conditions*, AMPK robustly inhibits the translocation of HuR to the cytosol.[196,197] Therefore, in these cases, increased AMPK activity directly contributed to the implementation of the senescent phenotype, as opposed to that observed when HuR is free to operate onto target mRNAs in the cytosol. As a consequence, AMPK activation can cause premature fibroblast *senescence* through mechanisms involving reduced HuR function.[190] During a *predominantly proteotoxic stress* (e.g., heat), however, inhibition of AMPK is beneficial to cell survival[57] in part because HuR is freed to stabilize SIRT1 thereby increasing HSF1 activity and the HSR. In fact, during a *bona fide* HSR, PP2A-mediated AMPK inhibition upregulates HSP70 expression at least partially through stabilizing its mRNA.[57]

The tumor suppressor kinase LKB1, which typically activates AMPK, may suppress HSF1 activity through Ser121 phosphorylation, preventing its nuclear translocation, DNA binding, and transcriptional activity.[198] To the contrary, HSF1 may reciprocally suppress AMPK by directly enforcing an inactive conformation. By physically evoking conformational switching of AMPK, HSF1 impairs AMP binding to the γ-subunits, enhances the PP2A-mediated dephosphorylation, and impedes the LKB1-mediated phosphorylation of Thr172, as well as retards ATP binding to the catalytic α-subunits.[56] Curiously, activation of PP2A in the brains of zebrafish and mice does reverse age-related behavioral changes in senescent neurons.[199] This is why, when there is *predominantly proteotoxic stress* (which releases HSF1 from chaperones), there is also a concomitant *blockade of AMPK*. *In toto*, the final destination and metabolic direction of the HuR-SIRT1 duet depends ultimately on the predominance of either *proteotoxic* or *metabolic stress* conditions, that is, caloristasis.[13]

Apart from the above findings, SIRT1 has also been shown to mitigate ER stress and insulin resistance triggered by saturated fatty acids in hepatocyte-like cells.[200] Additionally, alongside SIRT1, histone deacetylase 6 can detect protein aggregation and, through its ubiquitin-binding domain, relieve HSF1 from repression, resulting in the disassembly of inhibitory HSP90–HSF1 complexes, thereby enabling HSF1 activation in the presence of protein aggregation.[59] Resveratrol, a known inducer of SIRT1 metabolic action that obstructs NLRP3 inflammasome,[201] can shift the metabolism of mammals from a high-calorie diet to that of animals maintained on a standard diet. This is achieved by increasing insulin sensitivity, AMPK activity, and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which in turn increases the mitochondrial number and oxidative metabolism.[202,203] Moreover, SIRT1 activates PGC-1α through deacetylation, which stimulates the production and secretion of the myokine *Irisin*. This myokine acts in white adipose cells, both *in vitro* and *in vivo*, promoting a brown-fat-like phenotype through the stimulation of uncoupling protein-1 expression.[204] This link between calorie restriction, physical exercise, and protective energy-consuming oxidative metabolism is crucial for preventing metabolic syndrome and age-related diseases. Additionally, there is significant evidence highlighting the tight link between calorie restriction and exercise-induced HSR with metabolic stress, which is protective of metabolism. This is achieved by inducing chaperones during a healthy HSR, through the participation of anti-senescence SIRT1 pathways.[205] Therefore, the interaction between HuR and SIRT1 is of critical importance for the beneficial effects of the anti-inflammatory and anti-senescence HSR.

As a whole, these findings indicate that the HSR is progressively suppressed under conditions that surpass the capacity of the UPR to operate in favor of proteostasis. Subsequently, this sets in motion a continuous activation of the NLRP3 inflammasome, which, in a cascading effect, culminates in caspase-1-induced degradation of HuR. This degradation, in turn, results in a reduction in HSF1 expression and DNA-binding activity, ultimately leading to the dismantling of the HSR in such scenarios. Therefore, the Western lifestyle that constantly drives ER stress in adipose tissue culminates in UPR-elicited SASP *via* continuous activation of the NLRP3 inflammasome, which triggers a limitless suppression of the HuR–SIRT1–HSF1 axis and the consequent abolishment of the HSR. By downregulating HSF1 expression (and its DNA-binding activity) and the consequent anti-inflammatory HSR, SASP contributes to its own initiation and perpetuation.[179] Worse still, adipose tissue-emanated pro-inflammatory cytokines spread out through the organism, triggering SASP and suppression of the HSR in other tissues, as approached next.

SASP-mediated inflammatory response in non-adipose tissues: Evidence of systemic spread

Activation of the NLRP3 inflammasome in adipocytes plays a deciding role in the onset of various metabolic diseases, such as atherosclerosis and insulin resistance.[172] Interestingly, the activation of the NLRP3 inflammasome seems to act as a "danger sensor" in response to injurious stimuli that induce senescence, such as UVB irradiation or metabolic stress from high extracellular glucose.[206] In mononuclear cells of the immune system, including macrophages, NLRP3 inflammasome activation can be triggered by several signals, including glucose, palmitate, uric acid, ceramide, reactive oxygen species, and pancreatic islet amyloid polypeptide (IAPP).[207] In the context of Western HFD, the cleavage of SIRT1 induced by inflammation in adipose tissue is NLRP3 inflammasome-dependent.[208] Additionally, genetic ablation of NLRP3-comprising its components or caspase-1 leads to improved glucose tolerance and insulin sensitivity in HFD animals.[173,209] Curiously, metformin, a known inducer of AMPK activity, reduces NLRP3 protein expression and NLRP3 inflammasome activation in inflammatory macrophages,[210] thus operating as a proteostasis-saving kinase facilitating HSF1 activity and the HSR.

Obesity triggers the activation of the NLRP3 inflammasome in response to modified low-density lipoprotein particles, free fatty acids (particularly saturated), and intracellular cholesterol crystals, thereby modulating adipocyte differentiation and insulin sensitivity.[173,211] Actually, high cholesterol levels explain inflammation in the cardiovascular system due to NLRP3 inflammasome activation as well.[212] Notably, glucose directly stimulates caspase-1 activity,[207] adding yet another harmful consequence of hyperglycemia observed in diabetes mellitus of any type. NLRP3 inflammasome senses obesity-associated danger signals and contributes to obesity-induced inflammation and insulin resistance.[213] Hyperglycemia, palmitate, uric acid, LPS, and IAPP all prime the activation of inflammasomes in *target* cells, such as adipocytes, macrophages, hepatocytes, and the islet of Langerhans cells, promoting the start of caspase-1 mRNA expression. Moreover, the aggregates of IAPP are toxic to pancreatic β-cells and associated with the pathogenesis of T2DM. IAPP is also able to activate the NLRP3 inflammasome.[214] One concerning aspect of this narrative is the extended and copious activation of the NLRP3 inflammasome and the resulting dissemination of SASP, which eventually leads to cellular senescence instead of apoptosis in target cells. *Senescent cells resist apoptosis*, perpetuating chronic inflammation in tissues and sustaining chronic inflammatory diseases throughout the body.[172,206,215]

In the context of feminine endocrinology, estrogen is a potent inducer of the HSR.[10] Accordingly, estrogen depletion is linked to various inflammatory events in the CNS that are associated with NLRP3 inflammasome activation. However, in menopause, estrogen replacement can potentially alleviate this scenario by inhibiting NLRP3 inflammasome activation through the type β estrogen receptor pathway.[216] This has also been observed in reproductively senescent female rats.[217] Similarly, after global cerebral ischemia, a condition that causes significant oxidative stress and inflammation, local estrogen administration has been found to completely inhibit NLRP3 inflammasome activation and its associated inflammatory pattern.[218] Both estrogen and progesterone are known to modulate inflammasome activation and mediate anti-inflammation in the CNS.[219] These findings align with estrogen's anti-inflammatory effects, which mirror its anti-senescence actions. This lends support to the notion that some of estrogen's protective effects stem from its ability to maintain a robust HSR by disrupting the destructive cycle that diminishes HSF1 availability due to SASP and cellular senescence. Notably, heat shock treatment impedes the activation of the NLRP3 inflammasome,[220] thereby overcoming the destruction of HuR and tissue senescence associated with depressed HSF1 expression and low HSR. Hence, paradoxically, heat shock alone

can re-establish the HSR. These observations, alongside the findings described in Schroeder *et al.*,[221] suggest that heat treatment should be evaluated in menopausal women.

The association between metabolic stress-induced ER stress in adipose tissue and unresolved inflammation centered on the continuous activation of NF-κB members is a causal factor for the progression of metabolic diseases and has, in essence, the overwhelmed UPR being ultimately responsible for the continuous secretory profile of cytokines.[222,223] This "seeding" of inflammation takes place initially in the adipose tissue and then spreads throughout all body tissues that work as SASP-relaying points.[3] SASP-associated production of inflammatory cytokines, such as IL-1β, IL-6, IL-8, and IL-18, has been linked to a persistent DNA damage response[224] and, consequently, to an antiapoptotic senescence state. This perpetuates SASP-induced inflammation in all body tissues. There is an interaction between genes that regulate intermediate metabolism and those of the immune system, such as TNFα, leptin, adiponectin, resistin, interleukins IL-1β, IL-6, IL-8, IL-10, and C-reactive protein, just to cite a few examples, and all of them tend to impose a SASP and apoptosis-resistant senescence. In this regard, TNFα is known to activate signal transduction cascades, including some pathways strictly involved in inhibiting insulin action, such as the activation of JNKs. These kinases are particularly critical to insulin signaling in adipose tissue and striated muscle because they phosphorylate insulin receptors and other coupling proteins in serine residues, blocking the normal post-receptor insulin signals.[225] Activation of Toll-like receptor 4 in the intestine, due to nutritional and energy imbalances, leads to a change in the microbiota (gut dysbiosis) that also results in insulin resistance.[147,226] TNFα is strongly expressed in adipose tissue and skeletal muscle of obese individuals and, when administered in the circulation, induces insulin resistance.[227] Conversely, inhibition of the action of this cytokine in a model of obesity in rats leads to an increase of insulin-stimulated glucose uptake in adipose tissue.[228] TNFα reduces insulin signaling by inducing phosphorylation of IRS-1 in Ser307 in skeletal muscle, adipose tissue, and liver, while in the liver, this cytokine acts dependently on JNKs.[229,230] Furthermore, in a remarkable evolutionary innovation, a distinct *negative feedback mechanism* was introduced into insulin signaling to disrupt excessive downstream signal transduction *via* insulin receptors. This involves the phosphoinositide 3-kinase (PI3K)-dependent *activation of the very JNKs*, which disrupts insulin signals.[231] TNFα also imposes a similar inhibition over IRS-1 in muscle and fat tissues from obese rats.[230] Consequently, excessive insulin concentrations undeniably result in insulin resistance (i.e., *insulin-dependent insulin resistance*) and the generation of inflammatory signals in target tissues.

Since continuous activation of NLRP3 inflammasome-dependent and NF-κB-dependent production of pro-inflammatory cytokines become perpetual, pro-inflammatory kinases, such as JNKs, are rendered vigorously active inhibiting insulin signaling,[225] which could be avoided by the direct binding of HSP70 to them.[232] Studies from this laboratory with individuals undergoing bariatric surgery also show that the inflammatory process that begins in the adipose tissue spreads out to the liver, leading to a reduction in the expression of HSF1 and HSP70 with a significant increase in the expression of JNKs both in the liver and adipose tissue.[144]

As approached in the previous sections, GSK-3β, a serine/threonine protein kinase responsible for phosphorylating specific target serine and threonine amino acid residues, physiologically and constitutively inactivates HSF1 under normal cellular conditions.[233] Conversely, GSK-3β inactivation is contingent upon the PI3K pathway, effectively making it dependent on the insulin signaling pathway.[95] This elucidates why, during the development of insulin resistance, the mechanism for GSK-3β inactivation *via* AMPK and SIRT1 fails, resulting in the continuous suppression of HSF1 activity.[234] The reduced activity of the HSR pathway is directly linked to insulin resistance and the worsening of type 2 diabetes.[127,151] Therefore, it is crucial to monitor the temporal progression of chronic inflammatory diseases with regard to impaired HSR.[221]

Since both HuR and SIRT1 are recognized as anti-senescent and anti-inflammatory proteins,[10] senolytic therapies have been developed and are now in clinical trials. These therapies employ drugs (known as senolytics) that preferentially target senescent cells and selectively eliminate them by inducing apoptosis.[235,236] The aim is to stop the noxious ER-stress-SASP-inflammation vicious cycle and restore the physiological resolution of inflammation *via* HSR. Senescent cells can be dysfunctional, decreasing the survival rate even in young animals, whereas senolytics can enhance the lifespan of older ones.[237] In fact, combinations of FDA-approved senolytic drugs, such as quercetin and azithromycin, have been proposed for treating COVID-19 patients,[238] particularly those with chronic metabolic diseases of an inflammatory nature, which pose a significant threat to these patients.[120]

Although having underlying mechanisms not completely understood, aging may also be associated with deficient HSR. Aging is a complex process modulated by different molecular and cellular events, such as genome

instability, epigenetic and transcriptional changes, molecular damage, cell death, proliferative senescence, inflammation, and metabolic dysfunction.[9] Particularly, protein quality control (i.e., chaperone systems) tends to be negatively affected by aging, thus leading to cellular senescence in metabolic tissues and, as a consequence, to the increased dissemination of inflammation throughout the body. Of particular note is the age-dependent increase in inhibitory signals directed to HSPs, as well as hyperacetylation that is associated with a significant reduction in the activity of HSF1 in binding to DNA.[239] Hence, cellular senescence is a common link in the chain of chronic degenerative diseases, including during aging, because, as stated here, SASP-producing cells are extremely resistant to apoptosis and allow for a state of low-grade chronic inflammation all the way through the organism.[3,9]

A substantial body of research has explored the link between activating the HSR and extending longevity. In the nematode *C. elegans*, the post-reproductive stage is marked by a sudden decline in protein quality control.[9,240] Consequently, the capacity to respond effectively to heat stress becomes a crucial characteristic of *longer-lived animals*. In *C. elegans*, overexpression of the HSF1 orthologue, *hsf1*, in all tissues enhances resilience to stress and retards aging. Overexpression of *hsf1* in the CNS, on the other hand, is sufficient to enhance both protection against acute thermal stress and longevity,[241] possibly because this maneuver triggers cell-non-autonomous regulation of proteostasis through TCS.[34] Interestingly, a cold-sensitive strain of *Hydra oligactis* shifts from asexual budding to sexual reproduction when exposed to temperature stress (from 18 to 10 °C). This involves an upregulation not only of gametogenesis-related genes, but also of those related to *cellular senescence*, apoptosis, and DNA repair, as well as downregulation of genes involved in stem cell maintenance.[242] Sexual reproduction in Hydra species, as in vertebrates, is typically triggered or impeded by environmental cues indicative of proteotoxic or metabolic stress conditions (caloristasis). Under favorable environmental circumstances, Hydra primarily reproduces through asexual budding and does not exhibit noticeable gametogenesis.[243,244] Sexual reproduction, characterized by increased sperm and/or egg production from interstitial stem cells, can be induced in Hydra by environmental stressors such as food scarcity, crowding, or low temperatures, akin to conditions associated with the onset of winter.[245]

As organisms age, they frequently encounter functional decline, giving rise to conditions such as sarcopenia, atherosclerosis, heart and kidney failure, osteoporosis, macular degeneration, pulmonary insufficiency, and neurodegeneration, including Alzheimer's and Parkinson's diseases.[177] These conditions all share a common thread: senescence and the SASP. For a comprehensive understanding of the role of SASP, readers are encouraged to consult the thorough review by Tchkonia and colleagues,[246] which offers an in-depth perspective. Additionally, Zhu and collaborators have extensively detailed SASP's involvement in age-related chronic diseases.[224] It is noteworthy that Ames dwarf mice possess a 40–60% enhanced lifespan as compared to wild mice. Curiously, these animals concomitantly present an impressive insulin sensitivity in the skeletal muscle and liver.[247] This abnormal longevity is associated with altered methionine metabolism in these tissues leading to a complete modification of cysteine metabolism and enhanced antioxidant metabolism as compared to wild mice.[248] In contrast to typical aging patterns, it has been shown that the levels of HSF1 protein and mRNA in Ames dwarf mice actually increase as they age and can be further enhanced by exposure to stress! This suggests that exceptional longevity may be linked to compensatory and improved regulation of HSF1 as a response to age-related pressures that would otherwise suppress the heat shock axis,[239] as previously pointed out.[249] These findings corroborate once again that HSR is strongly linked to energy metabolism,[16] as prophesied by Ritossa in his first papers on the effects of heat shock on polytene chromosomes of *Drosophila spp.*[250–252] and recently confirmed.[13,58,59]

Cardiovascular dysfunctions, which are prevalent during aging, are closely related to other metabolic diseases, particularly in terms of depressed HSR, which is always evident. Metabolic changes caused by over-utilization of preadipocytes lead to obesity, which is accompanied by a state of cellular senescence that impairs an effective HSR when needed.[175,179,246] Pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and IL-18, which are produced as a result of SASP-related cytokine storm, spread throughout the body, promoting insulin resistance[253] and negatively affect blood vessels and the heart, impairing both tissue repair and the efficiency of antioxidant systems.[254–258] Ultimately, this leads to low-grade sterile inflammation that cannot be cured.[3] Obesity not only causes insulin resistance and impairs the HSR in blood vessels, but it also heightens vascular senescence and susceptibility to ischemic injury, increasing the likelihood of peripheral and cerebral ischemic episodes. Moreover, cellular senescence of vascular smooth muscle cells stimulates atherosclerosis (an essentially inflammatory disease) and plaque vulnerability.[176,256,259,260]

Although senescence can initially cause growth arrest (which could impede the dissemination of inflammatory cells), it also triggers the production of various growth factors and pro-inflammatory cytokines

that contribute to SASP.[255,261] As time passes, the SASP can lead to chronic oxidative stress[262] and low-grade inflammation in the vascular intima-media layers, which is a hallmark of atherosclerosis.[263] While vascular HSR may indeed increase during the early phases of atherogenesis,[264–267] it eventually collapses during later stages, contributing to the loss of proteostasis, deregulated nutrient sensing, and plaque instability.[254,268] In summary, although senescence initially could have a desirable (antiproliferative) effect on the vascular wall and adipose tissue by causing growth arrest, it ultimately leads to the production of harmful growth factors and pro-inflammatory cytokines that cause chronic oxidative stress and inflammation. As a result, cellular senescence (which occurs during organismal aging) can cause DNA damage and weaken antioxidant responses, leading to increased vascular inflammation and worsening of atherosclerosis.[145,256] It is noteworthy that heat stress has long been shown to reduce free-radical-mediated oxidative stress in the heart[269] whereas HSP70 suppresses neuroinflammation caused by protein aggregation.[270,271] Actually, pharmacological treatments based on A-type cyPGs, which are powerful HSR inducers, have been shown to prevent the progression of atherosclerotic lesions in mice.[272] In addition, taking into account that oxidative stress disrupts the interaction between HuR and SIRT1 mRNA due to NLRP3 inflammasome activation,[170] physiological maneuvers intended to remediate the HSR through heat treatments[139,156,273–275] emerge as alternatives to pharmacological interventions, as approached in Schroeder *et al.*[221]

Aging is considered the strongest independent risk factor for developing atherosclerosis.[276] It induces vascular smooth muscle cell senescence, which triggers peripheral monocytosis and macrophage recruitment within atherosclerotic lesions.[277] Similarly, aging-associated senescence in the heart reduces tissue capacity to respond to stress and protect against injury.[278] Cellular senescence plays a central role in the etiology and progression of various chronic inflammatory diseases, including atherosclerosis, hypertension, diabetes, osteoarthritis, and Alzheimer's disease,[224] as it parallels the development of dysfunction in different tissues.

As a whole, the failure of the HSR due to SASP can have serious consequences for our health, including an increased risk of chronic degenerative diseases of an inflammatory nature. However, there is hope. By making simple lifestyle changes and incorporating practices such as regular exercise, sauna, and hot tub use, we can help to reactivate and support the body's HSR. Sauna and hot tubs are particularly useful for those with limited access to exercise. These methods have been used for centuries and have been shown to have significant health benefits. Additionally, pharmacological approaches may also hold promise for activating the HSR in those with chronic diseases. These specific points and the clinical significance of evaluating the HSR using cost-effective and accessible tools are presented in the review article by Schroeder *et al.*[221]

Conclusion

Delving into the perplexing interplay between the modern Western lifestyle and the failure of the HSR to orchestrate the resolution of inflammation is akin to savoring the nuances of a symphony. Because of this, we decided to stay seated in the front row of this intricate performance, where every note and crescendo reveals the profound implications of caloristasis imbalances on the grand stage of human health.

In the dazzling tapestry of metabolic management, metazoans possess a remarkable arsenal to counteract the perils of energy scarcity, orchestrating a symphony of physiological mechanisms at both the macro and micro scales. At the macro scale, the SNS acts as the conductor of homeostasis, directing the harmonious glycogenolysis in muscle and liver tissues. Glucagon, growth hormone, and corticotropin-releasing hormone chime in as key instrumentalists. At the micro-scale, AMPK, GSK-3β, SIRT1, and HuR oversee the intricacies of oxidative metabolism. However, in remarkable contrast, when it comes to managing abundant energy reserves, there is a singular dominant conductor: the insulin pathways. Additionally, and quite remarkably, evolution introduced an innovation utilizing a unique negative feedback mechanism in insulin signaling to prevent excessive downstream signal transduction through insulin receptors. This mechanism involves PI3K-dependent activation of JNKs, effectively halting insulin signals. Therefore, excess insulin concentrations undoubtedly lead to insulin resistance and inflammatory signals originating from target tissues. In other words, the blueprint of evolution did not anticipate the need to navigate the challenges posed by our contemporary societies' abundance of energy surplus. This, in part, elucidates the extraordinary difficulty our organisms encounter in combating the conditions that lead to caloristasis imbalances, ultimately instigating the inception of chronic inflammatory symphonies by stifling the melodies of the HSR.

Chronic degenerative diseases, with their inflammatory overtures, emerge from the discord of caloristasis imbalances, orchestrating the debilitating SASP and silencing the HSR's harmonious resolution.

While the ancient score of human genetics and metabolism remains relatively unaltered over millions of years, our modern lifestyle, marked by surplus energy intake and insufficient physical activity, exacerbates this dissonance. Rising rates of obesity and diabetes, along with their associations with a host of other chronic maladies, create a chorus of progressive failure to resolve inflammation.

During the grand overture of vertebrate evolution, inflammation evolved as a defense mechanism against harmful microorganisms and for tissue repair, with two distinct phases: initiation and resolution. It is precisely the resolution phase that is increasingly compromised, leading to low-grade inflammation underlying these chronic diseases. The HSR, an evolutionarily conserved pathway, plays a critical role in resolving inflammation by restoring proteostasis in cells. However, the constant activation of UPR turns on inflammasomes, particularly NLRP3, leading to the degradation of HuR and a decline in HSR effectiveness. Proliferative senescence triggered by continuous NLRP3 activation imposes SASP, which perpetuates chronic inflammation, especially in adipose tissue. Senescent adipose cells, in turn, resist apoptosis, further propagating inflammation to various tissues. Consequently, metabolic diseases like insulin resistance, atherosclerosis, and neurodegeneration emerge.

To restore the symphony of health, further research must uncover the hidden notes of HSR impairment and compose more efficacious therapies. The crescendo of chronic inflammation and its accompanying infirmities resonates globally, emphasizing the urgency of harnessing the symphonic power of the HSR in this enduring battle.

"Quæ medicamenta non sanat æ ferrum sanat. Quæ ferrum non sanat æ ignis sanat. Quæ vero ignis non sanat æ insanabilia existimare oportet.

That which drugs fail to cure the scalpel can cure. That which the scalpel fails to cure heat can cure. If heat cannot cure, it must be determined to be incurable."

(Aphorisms of Hippocrates, by Elias Marks, from the Latin version of Verhoofd, Collins & Co., New York, 1817)

Funding and support This work has been supported by the State of Rio Grande do Sul Foundation for Research Support (FAPERGS/Decit/SCTIE/MS/CNPq/SESRS n. 03/2017-PPSUS #17/2551-0001424-3 to MK and #19/2551-0001713-8 to PIHdB) and the Brazilian National Council for Scientific and Technological Development (CNPq, process #303853/2017-4 to PIHdB, process #307926/2022-2 to TGH and process # 302959/2020-3 to MK). Financial support from CAPES (Coordination of Superior Level Staff Improvement) is also acknowledged.

Author contribution PIHdB conceptualized the paper, prepared the initial draft, and oversaw its finalization. All the authors were involved in co-writing this work. PIHdB prepared the figures. All the authors have read and agreed to the submitted and published versions of the manuscript.

Data availability statement Data will be made available on request.

Declarations of interest The authors declare no conflict of interest and no competing interests such as consultancies, financial involvement, patent ownership, etc. in relation to the work described. CNPq, FAPERGS, and CAPES (the funding organisms) had no involvement in the propositions presented in this manuscript.

Acknowledgments The authors are indebted to Dr. Maria Inês Lavina Rodrigues for her invaluable technical support during the experiments that support many of the findings described herein. The authors apologize for not including many important primary studies in their text due to space constraints. Their goal was to ensure a smooth flow of information throughout the text.

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Capítulo III

Título: Heat shock response during the resolution of inflammation and its progressive suppression in chronic-degenerative inflammatory diseases

Periódico: Cell Stress and Chaperones

Situação: Publicado em fevereiro de 2024.

(disponível em: <https://doi.org/10.1016/j.cstres.2024.01.005>).

Cell Stress and Chaperones (2024) 29:66–87
https://doi.org/10.1016/j.cstres.2024.01.005

REVIEW

Resolution of inflammation in chronic disease *via* restoration of the heat shock response (HSR)

Helena Trevisan Schroeder[a,1] · **Carlos Henrique De Lemos Muller**[b,2] · **Thiago Gomes Heck**[c,3] · **Mauricio Krause**[b,4] · **Paulo Ivo Homem de Bittencourt Jr**[a,*,5]

Received: 15 September 2023 / Revised: 28 January 2024 / Accepted: 29 January 2024

Abstract

Effective resolution of inflammation *via* the heat shock response (HSR) is pivotal in averting the transition to chronic inflammatory states. This transition characterizes a spectrum of debilitating conditions, including insulin resistance, obesity, type 2 diabetes, nonalcoholic fatty liver disease, and cardiovascular ailments. This manuscript explores a range of physiological, pharmacological, and nutraceutical interventions aimed at reinstating the HSR in the context of chronic low-grade inflammation, as well as protocols to assess the HSR. Monitoring the progression or suppression of the HSR in patients and laboratory animals offers predictive insights into the organism's capacity to combat chronic inflammation, as well as the impact of exercise and hyperthermic treatments (e.g., sauna or hot tub baths) on the HSR. Interestingly, a reciprocal correlation exists between the expression of HSR components in peripheral blood leukocytes (PBL) and the extent of local tissue proinflammatory activity in individuals afflicted by chronic inflammatory disorders. Therefore, the Heck index, contrasting extracellular 70 kDa family of heat shock proteins (HSP70) (proinflammatory) and intracellular HSP70 (anti-inflammatory) in PBL, serves as a valuable metric

Abbreviations: **AMPK**, 5'-adenosine monophosphate-activated protein kinase; **COVID-19**, coronavirus disease-2019; **COXIBs**, inhibitors of cyclooxygenase (prostaglandin endoperoxide H synthase); **CRP**, C-reactive protein; **CVD**, cardiovascular disease(s); **ER**, endoplasmic reticulum; **GSK-3β**, glycogen synthase kinase-3β; **HbA1c**, % of glycated hemoglobin $A1_C$; **HBP**, hexosamine biosynthetic pathway; **HFD**, high-fat diet; **HOMA-IR**, homeostasis model assessment-insulin resistance index; **HSF1**, heat shock transcription factor-1; **HSP**, heat shock protein; **HSP70**, the 70 kDa family of heat shock proteins; **iHSP70**, intracellular HSP70; **eHSP70**, extracellular HSP70; **HSR**, heat shock response; **HuR**, human antigen R, a.k.a. ELAV-1, for *E*mbryonic *L*ethal, *A*bnormal *V*ision, Drosophila, Homolog-Like protein-1; **IRE1**, Inositol Requiring Enzyme-1, also known as Endoplasmic Reticulum-to-Nucleus Signaling-1, ERN1; **JNK**, c-Jun *N*-terminal kinase; **NAFLD**, non-alcoholic fatty liver disease; **NF-κB**, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; **NLRP3**, NOD-like receptor pyrin domain-containing protein-3 inflammasome; **NSAIDs**, non-steroid anti-inflammatory drugs; **PBMC**, peripheral blood mononuclear cells; **PGs**, prostaglandins; **cyPGs**, cyclopentenone prostaglandins; **PERK**, *P*rotein kinase-like *ER K*inase; **SASP**, Senescence-Associated Secretory Phenotype; **SIRT1**, NAD^+-dependent deacetylase sirtuin-1; **T1DM**, type 1 diabetes mellitus; **T2DM**, type 2 diabetes mellitus; **TLR**, Toll-like receptor; **TNFα**, tumor necrosis factor-α; **UPR**, unfolded protein response

[1]0000-0003-4000-3671
[2]0000-0003-0205-7775
[3]0000-0002-1242-5423
[4]0000-0001-9814-742X
[5]0000-0003-1907-3341

* Paulo Ivo Homem de BittencourtJr
pauloivo@ufrgs.br

[a] Laboratory of Cellular Physiology (FisCel), Department of Physiology, Institute of Basic Health Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil

[b] Laboratory of Inflammation, Metabolism and Exercise Research (LAPIMEX), Department of Physiology, ICBS, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil

[c] Post Graduate Program in Integral Health Care (PPGAIS-UNIJUÍ/UNICRUZ/URI), Regional University of Northwestern Rio Grande Do Sul State (UNIJUI) and Post Graduate Program in Mathematical and Computational Modeling (PPGMMC), UNIJUI, Ijuí, Rio Grande do Sul, Brazil.

Published online: 01 February 2024

for HSR assessment. Our laboratory has also developed straightforward protocols for evaluating HSR by subjecting whole blood samples from both rodents and human volunteers to *ex vivo* heat challenges. Collectively, this discussion underscores the critical role of HSR disruption in the pathogenesis of chronic inflammatory states and emphasizes the significance of simple, cost-effective tools for clinical HSR assessment. This understanding is instrumental in the development of innovative strategies for preventing and managing chronic inflammatory diseases, which continue to exert a substantial global burden on morbidity and mortality.

Keywords HSP70 · Heat shock response · Low-grade inflammation · Heck index of HSP70 · Type 2 diabetes mellitus

Introduction

Inflammation is a highly conserved physiological response that evolved to eliminate microorganisms or facilitate tissue recovery during an aseptic injury. While these responses are typically acute, a Western lifestyle based on insufficient physical activity and surplus energy consumption predisposes to progressive suppression of the physiological resolution of inflammation through the heat shock response (HSR). This is mainly because lifestyle-elicited energy imbalances overwhelm the endoplasmic reticulum (ER) of adipose tissue leading to ER stress and eventually to the unfolded protein response (UPR). However, the UPR has an inflammatory branch through the protein kinase-like ER kinase-elF2α-ATF4-CHOP and inositol requiring enzyme-1–c-Jun *N*-terminal kinase (JNK) pathways that relay inflammatory signals throughout the body.[1] As discussed by Schroeder and colleagues,[2,3] continuous activation of UPR downstream pathways culminates in uninterrupted activation of NOD-like receptor pyrin domain-containing protein-3 (NLRP3) inflammasome in adipose tissue, which leads to the production of activated caspase-1. Caspase-1, in turn, progressively degrades human antigen R (also known as embryonic lethal, abnormal vision, Drosophila, homolog-like protein-1), an mRNA-binding protein that is indispensable for the expression and activation of the heat shock transcription factor-1 (HSF1). Therefore, chronic ER stress of adipose tissue gradually impedes the resolution of inflammation *via* the HSR in several tissues, not just in the adipose tissue. This leads to the establishment of chronic low-grade inflammation that accompanies chronic degenerative conditions such as insulin resistance, obesity, type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD), cardiovascular diseases (CVDs), and neurodegenerative diseases (e.g., Alzheimer's, Parkinson's, Huntington's).

Although chronic degenerative diseases of an inflammatory nature take place precisely because the HSR becomes jeopardized as long as noxious stimuli (e.g., Western lifestyle) do not cease to activate NLRP3 inflammasomes, the HSR can be nonetheless re-established through strategies that are known to trigger the activation of HSF1 and 70 kDa family of heat shock proteins (HSP70) expression. If this could appear paradoxical at first glance, heat shock (HS) (i.e., fever, thermotherapy) or even exercise, by different mechanisms, are able to dismantle NLRP3 inflammasome activation, resuming the HSR and thereby relieving the above chronic inflammatory conditions.

In the present manuscript, we review current physiological, pharmacological, and nutraceutical approaches aimed at rearming the HSR in chronic inflammatory conditions. We also discuss simple and cost-effective tools used to clinically evaluate the progression or suppression of the HSR in humans and laboratory animals.

Physiological approaches

The physiological activation of the HSR primarily hinges on two key factors: proteostasis-threatening conditions and metabolic stress. These factors can be mutually exclusive, with the prevalence of one over the other dependent on a delicate metabolic balance referred to as "*caloristasis,*"[4] as elaborated upon in.[2] For instance, an increase in the bodily core temperature represents a proteostasis-threatening situation, as heat can alter protein conformation, potentially leading to the formation of toxic protein aggregates that trigger an inflammatory response. Therefore, hyperthermic treatments such as saunas or hot tub baths can robustly activate the HSR throughout the body. Additionally, metabolic signals directed toward energy-conserving pathways, such as 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) or NAD^+-dependent deacetylase sirtuin-1 (SIRT1), can also induce a genuine HSR. This is evident in the case of physical exercise, where AMPK activation may result from decreased intracellular glycogen levels, directly correlating with elevated mRNA and protein levels of HSP72.[5] AMPK activation in such cases inhibits glycogen synthase kinase-3β, which consistently and constitutively represses HSF1 activity,[6] thereby mitigating a bona fide HSR. Notably, HSR activation during

exercise is not solely reliant on increased body or muscle temperature.[7] However, if cellular metabolic sensors perceive that metabolic stress poses a greater threat to overall bodily homeostasis than the activation of the HSR to correct misfolded proteins (an energy-intensive process, as core chaperones like HSP70 or HSP90 utilize ATP to preserve protein function), AMPK may impede HSF1 activation.[8,9] In essence, whether the proteostasis-preserving HSR is triggered or not hinges on the delicate balance governing caloristasis.

With these reservations duly noted, (re)activating the HSR can often be achieved through fever, or rather, by deliberately not suppressing fever in many situations may prove beneficial.[2] Remarkably, fever adeptly orchestrates antimicrobial defenses and contributes to the regulation of inflammation and tissue healing, a phenomenon observed even in cold-blooded vertebrates, where there exists a discernible selectivity in the immune responses induced by fever. In essence, fever not only suppresses inflammation but also substantially amplifies the process of wound repair, as detailed in a recent study by Haddad *et al.,*[10] extending its effects across the entire metazoan kingdom.

Fever, in its broad sense, is thought to have evolved in modern animals approximately 600 million years ago.[11] It is not limited to homeothermic mammals and birds; many poikilothermic animals, including lower vertebrates, arthropods, and annelids, can also increase their core temperature in response to infection or injury through a variety of behaviors.[10,12] However, generating a fever is a complex response that is very metabolically costly.[13] In humans, producing fever may require a six-fold increase in metabolic rate, while maintaining core temperature at febrile levels may demand a 12% increase in metabolic rate per 1 °C increase.[11] In essence, despite the significant energy expenditure it demands from an organism, evolution has consistently favored fever as an indispensable mechanism for maintaining homeostasis. Indeed, fever, or the sustained elevation of body temperature, emerges as a last resort and a natural therapeutic tool with the potential to curtail excessive cytokine production in critically septic patients and those afflicted with chronic inflammatory metabolic disorders, as highlighted by Heck *et al.*[13] Consequently, indiscriminately suppressing fever through the use of antipyretic medications seems, to say the least, unwise. This consideration becomes even more crucial when considering the disruption caused by inhibitors of cyclooxygenase-2 (prostaglandin endoperoxide H synthase-2; COXIBs) and traditional nonsteroid anti-inflammatory drugs (NSAIDs) in the physiological resolution of inflammation orchestrated by the HSR, as expounded upon in the works of Schroeder *et al.*[2,3]

Fever works as an integrative response regulating the induction and resolution phases of acute inflammation.[10,11] A rise in core temperature of about 2–3 °C initiates the HSR,[11] which provides physiological resolution of inflammation at the same time that it impedes intracellular protein aggregation because the HSR produces anti-aggregative protein chaperones, such as HSP70.[14,15] Structural changes in the plasma membrane during the establishment of fever also participate in HSF1 activation.[16]

Because HSP70s are stress-inducible proteins activated by both hyperthermia and hypothermia,[17,18] physical approaches that can elevate local or central temperature may be employed to stimulate the HSR. As stated above, exercise is a powerful activator of HSR through mechanisms that include intracellular metabolic challenges (e.g., increase in AMP/ATP and NAD^+/NADH ratios), elevated sympathetic tonus to striated muscle, adipose tissue, and pancreatic islets, as well as slight increases in core temperature itself. This explains at least partially the well-known beneficial impacts of exercise.[19–28] Nonetheless, besides exercise, hyperthermic treatment itself has demonstrated innumerable beneficial effects for the body by reducing fat deposition, alleviating insulin resistance, and decreasing total body mass.[25]

Although hyperthermic treatment was well-known to the ancient Romans and by Hippocrates himself, the first indication for the therapeutic use of thermal therapy occurred, incredible, only in 1999, when Professor Philip L. Hooper investigated the ability of hot-tub therapy (37.8–41.0 °C water temperature with average oral temperature rise of 0.8 °C) to reduce blood glucose in diabetic humans.[29] Interestingly, Professor Hooper's primary curiosity was not the HSR itself. Instead, he and his colleagues conjectured that the effects of partial immersion in a hot tub could mimic the known beneficial effects of exercise by increasing blood flow to skeletal muscle. Actually, most of the beneficial effects of thermotherapy in the muscle are a consequence of sympathetic stimulation of the muscle vasculature (so, vasoconstriction; not vasodilation) in order to prevent a dangerous fall in cardiac output due to cutaneous vasodilation that follows immersion in hot water.[25,30] Thus, thermotherapy could in fact be of value in treating diabetic people, as one of the features of insulin resistance is the decreased expression of HSP72 in the skeletal muscle of T2DM patients.[31]

Hot tub treatment (40 °C, 30-min sessions) has been shown to improve life quality and hemodynamic function in chronic heart failure patients, including postmenopausal women.[32] In our laboratory, for instance, by using a mouse model of atherosclerosis (a typical inflammatory disease), we applied HS treatment with

Table 1
Characteristics of studies in humans employing heat therapy to increase HSP70.

Study	Sample size	Condition	Treatment	Duration (week)	Frequency (times/week)	HSP70 result	Main findings
Ref.[36]	20	Healthy young	Heat therapy	8	4–5	↑ PBMC	↓ NF-κB in PBMC, superoxide production and MnSOD
Ref.[37]	20	Healthy young	Heat therapy leg	1	6	↑ Muscle	↑ PGC-1α and mitochondrial respiratory capacity
Ref.[38]	18	Overweight adults	Hot water immersion	2	5	↓ Plasma ↔ monocytes	↓ FBG and insulin
Ref.[42]	40	Metabolic syndrome or T2DM middle-aged	MES + HS	12	4	↑Expression in monocytes	↓ Visceral adiposity, wc, BP, FPG, HOMA-IR, TNF-α and CRP. ↑ adiponectin.
Ref.[43]	60	Obese with T2DM middle-aged/elderly	MES + HS	12	2, 4, or 7	↑Expression in monocytes	↓ Visceral adiposity, wc, BMI, FPG, HOMA-IR, HbA1c, TNF-α and CRP. ↑ adiponectin.

Abbreviations used: BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; FPG, fasting plasma glucose; HbA1c, % of glycated hemoglobin A1C; HOMA-IR, homeostasis model assessment-insulin resistance; HS, heat shock; HSP70, 70 kDa family of heat shock proteins; MES, mild electrical stimulation; MnSOD, manganese superoxide dismutase; NF-κB, nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family; PBMC, peripheral blood mononuclear cells; T2DM, type 2 diabetes mellitus; TNF-α, tumor necrosis factor-alpha; wc, waist circumference.

hot water immersion (41.5 °C for 15 min) once a week over 8 weeks and observed an impressive decrease in the animal mortality rate, significant improvement of the established vascular disease, increased blood flow, amelioration of ultrasonographic parameters, along with reversal of the depressed HSR.[33] Heat treatment, even in the fever-like range, blocks the NLRP3 inflammasome-dependent senescence-associated secretory phenotype (SASP),[34] re-establishing the human antigen R-SIRT1-HSF-1 downstream pathways. Therefore, HSR, through HS may, paradoxically *a priori*, reactivate its major biochemical pathway, namely, the HSR.

Whatever the mechanisms involved, hyperthermic manipulations have been employed therapeutically in complications related to obesity and T2DM,[24–26,29,30] especially for people to whom access to physical exercise is initially difficult.[35]

While the existing body of research on HSP70 levels in individuals undergoing heat therapy is limited, these studies, as summarized in Table 1, yield promising results. In one study involving young sedentary adults who underwent 4–5 sessions of hot water immersion per week for 8 weeks, an increase in intracellular HSP70 (iHSP70) coincided with a reduction in nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family (NF-κB) activation in peripheral blood mononuclear cells (PBMCs).[36] Similarly, in a comparable group of volunteers exposed to six consecutive sessions of pulsed short-wave diathermy-induced heat stress, a significant rise in muscular HSP70 levels was observed, which correlated with enhanced mitochondrial function.[37] However, sedentary overweight adults undergoing 2 weeks of hot water immersion (a total of 10 sessions) did not experience changes in HSP70 content within monocytes. Nevertheless, they exhibited a decrease in plasma HSP70 levels concurrent with improvements in fasting glucose and insulin.[38] This observation holds significance because elevated levels of extracellular HSP70 (eHSP70) are linked to low-grade inflammation and the exacerbation of T2DM.[25,39,40] Furthermore, a study involving both older and younger vervet monkeys, subjected to biweekly thermal hydrotherapy sessions over 5 weeks, revealed a notable correlation between the elevation of HSP70 levels in skeletal muscle and a reduction in fasting glucose levels.[41] These findings raise the possibility that the beneficial effects observed in thermotherapy may extend to individuals with various chronic low-grade inflammatory conditions.

Another series of studies examined the effects of mild electrical stimulation (MES) combined with HS (MES + HS) on individuals with chronic low-grade inflammation, including those with metabolic syndrome and T2DM.[42,43] The MES + HS treatment involved placing a device with electrodes on both the front and back of the abdomen, delivering a direct current at 1.4 ± 0.1 V/cm (55 pulses/s) and heat (42 °C), each pulse lasting 0.1 ms. After 4 weeks of MES + HS applied four times a week for 60 min, the studies revealed an increase in HSP70 expression in monocytes. Furthermore, following 12 weeks of treatment, significant improvements were observed in body composition, blood pressure, fasting blood glucose levels, the homeostasis model assessment-insulin resistance (HOMA-IR) index, and inflammatory markers.[42] In another study by the same research group, they investigated the effects of MES + HS in obese individuals with T2DM over 12 weeks.[43] Participants were divided into three groups, each receiving the treatment at different frequencies: two times a week (group 1), four times a week (group 2), and seven times a week (group 3), all for 60 min per session. Following the treatment, an increase in HSP70 expression in monocytes was observed in all groups. Moreover, when comparing all groups with their baseline measurements, significant reductions were noted in visceral adiposity, waist circumference, body mass index, fasting blood glucose, HOMA-IR, % of glycated hemoglobin A1C (HbA1c), and proinflammatory markers. Conversely, adiponectin levels increased. Notably, higher treatment frequencies were associated with more pronounced reductions in HOMA-IR, HbA1c, and diastolic blood pressure, indicating an improvement in the parasympathetic to sympathetic tone.[43] In summary, these studies consistently demonstrated favorable outcomes in terms of body composition, blood glucose regulation, and inflammatory markers when applying HS to specific body regions in conjunction with MES. Additionally, this treatment led to enhanced basal expression of HSP70 in monocytes among individuals with metabolic disorders.

In addition to hot tubs, saunas represent another form of hyperthermic treatment with a proven safety profile across various clinical conditions. Beyond its metabolic benefits, sauna heat therapy has been shown to induce an anti-senescent effect on blood vessels.[30] This effect is mediated by the HSR, which leads to the disinhibition of key regulators such as AMPK, SIRT1, and endothelial nitric oxide synthase (eNOS) expressions.[44] These findings strongly imply that the decreased risk of sudden cardiac death, fatal coronary heart disease, fatal CVD, and overall mortality associated with increased sauna bathing frequency in humans[45] may be attributed to the heightened HSR achieved through heat therapy. Notably, sauna therapy is now recognized as beneficial for CVD patients by improving endothelial function[46] and overall cardiovascular health,[47,48] even among those with heart failure.[30]

While sauna is contraindicated *a priori* for patients with unstable angina pectoris and recent myocardial infarction, it is generally considered safe for most coronary heart disease patients, particularly those with a history of stable angina pectoris and a remote history of myocardial infarction.[49] Furthermore, sauna therapy has been demonstrated as safe during uncomplicated pregnancies among healthy women, as affirmed by numerous studies.[49]

Table 1 provides an overview of the outcomes from various studies investigating the effects of hyperthermic treatment on the HSR in obese, T2DM, and healthy individuals.

While sympathetic tonus to the musculature may be a principal mechanism for triggering the HSR,[25,30] many of the health-benefiting effects of heat treatment, especially when applied locally, rely on the localized production of the vasodilatory and gaseous free radical nitric oxide (NO). NO, in fact, serves as a potent activator of HSP70 expression[50–53] and the accompanying HSR because it induces mild oxidative stress, ultimately activating HSF1 through disulfide bond formation.[15,30] Consequently, this slight redox imbalance induced by NO acts as a catalyst for the HSR.[54] Furthermore, a portion of the cytoprotective and anti-inflammatory effects attributed to estrogen-mediated HSR[55–58] can be linked to estrogen's capacity to stimulate NO production in various tissues. In alignment with this concept, estrogen's protective effects against cerebral ischemia[59] and various other types of injuries[60] are primarily ascribed to its influence on HSP70 expression. An inherent deduction drawn from these observations is that enhancing the production of NO within the vascular wall may furnish a cytoprotective HSR in the context of CVD induced by diabetes, as emphasized by Hooper *et al.*[22]

Rearming the HSR through the manipulation of gut microbiota

The HSR is influenced by the gut microbiota, with a direct relationship to the production of NO, a powerful HSR inducer, as stated above. The gut microbiota has the capacity to generate NO from nitrate,[61,62] enhancing the HSR in the intestinal epithelium. However, excessive NO production in the gut can be detrimental, leading to enterocyte apoptosis and hindering epithelial restitution processes.[63] While the precise balance of NO remains uncertain, understanding the regulation of HSR at the intestinal level is crucial. This is because the gut microbiota ecosystem impacts low-grade inflammation and related chronic inflammatory diseases,[64,65] and obesity induces alterations in gut microbial metabolism linked to the proinflammatory senescence-associated secretory phenotype (SASP) and tissue senescence.[66] Furthermore, the fermentation of carbohydrate prebiotics by the gut microbiota modulates NOS/NO pathways and NO-producing bacteria, simultaneously mitigating systemic endothelial dysfunction.[67,68] Additionally, evidence suggests that the enteric microbiota is a key determinant of immunity through the modulation of HSP production in intestinal epithelial cells.[69]

Gut microbiota plays a pivotal role in regulating the immune, metabolic, and even eating-behavioral aspects of mammals,[70–74] and the *diversity* of commensal microbiota is closely tied to an organism's health.[75] For instance, in conditions such as obesity and T2DM, the interaction between gut microbiota and the host plays a pivotal role in the development of metabolic disorders. Excessive dietary fat intake, for example, heightens systemic exposure to potentially proinflammatory free fatty acids and their derivatives, elevating plasma lipopolysaccharide (LPS) levels, a phenomenon termed "*metabolic endotoxemia*."[64,65,76] Imbalances in the distribution of gut microbiota taxa further contribute to the disruption of gut barrier integrity.[76] Conversely, adherence to a Mediterranean diet has been linked to various health benefits, including reduced mortality, lower rates of obesity, T2DM, low-grade inflammation, cancer, Alzheimer's disease, and depression. Additionally, recent studies suggest that following a Mediterranean diet may delay the onset of Crohn's disease.[77]

A reduction in gut microbiota species *diversity* has been observed in obesity and T2DM,[78,79] along with an imbalance between beneficial and harmful species.[80] Such alterations can also result from pathogenic infections, as seen in COVID-19.[81] Notably, diabetic individuals exhibit an increased presence of Proteobacteria,[82–84] a pattern also associated with conditions like Crohn's disease and colitis.[85] Although Firmicutes and Bacteroidetes represent 90% of gut microbiota, the dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia.[86] In addition, the relative proportion of Bacteroidetes genera has long been known to be decreased in obese people in comparison with lean people, while this proportion increases with low-calorie diet-induced weight loss.[87]

Gut microbiota has a preponderant role in insulin resistance.[88] The composition of the microbiota is even proposed as a marker for diseases or disease stages, such as fibrosis in NAFLD patients.[89] Furthermore, probiotics, live microorganisms used to promote health,[90] have demonstrated potential benefits as adjunctive treatment of chronic inflammatory diseases. For instance, the yeast probiotic *Saccharomyces boulardii* changes gut microbiota

and reduces hepatic steatosis, low-grade inflammation, and fat mass in obese and T2DM db/db mice.[91] Probiotics are promising also for the treatment of humans with T2DM.[92]

Nutritional imbalances and shifts in gut microbiota are key contributors to obesity and insulin resistance. Dysregulation of gut microbiota due to the Western lifestyle results in defects in intestinal tight junctions, leading to chronic endotoxemia and exacerbating systemic inflammation.[76] This mechanism is intricately linked to an increase in circulating LPS, as demonstrated in the seminal work by Cani and colleagues, which revealed that a high-fat diet alters gut microbiota and leads to elevated LPS levels and bacterial translocation into the bloodstream in mice.[64,65] In addition to the presence of plasma LPS and bacteremia, this situation is unfortunate as the increased prevalence of bacterial LPS-producing microbes results in LPS-induced metabolic endotoxemia. This condition triggers obesity, insulin resistance, and diabetes by disrupting insulin sensitivity and weight control through the LPS/CD14 system.[64] Subsequent studies have consistently reaffirmed these findings.[91,93,94]

Alterations in intestinal barrier permeability and heightened nutrient availability[95,96] are also involved. Dietary fat directly diminishes the distribution and expression levels of tight junction components, including occludin, E-cadherin, claudin, and junctional adherens molecule, leading to the disruption of intestinal permeability.[97] Furthermore, high intake of γ-linolenic (ω-6) and docosahexaenoic (ω-3) acids, typical in the Western diet, may also modulate intestinal permeability by stimulating intracellular signaling pathways *via* protein kinase C pathways.[97] Altogether, these factors contribute to low-grade inflammation, fostering insulin resistance independently of weight gain.[98] LPS activates toll-like receptor 4 (TLR4), triggering the JNK pathway, which can obstruct insulin activation downstream to insulin receptor phosphorylation.[99] Gut microbiota imbalances may also induce insulin receptor *S*-nitrosation by boosting inducible NOS activity, which is NF-κB-dependent.[100,101]

To address this inflammatory condition, the preservation of the intestinal barrier integrity is paramount, and the HSR appears to play a pivotal role. HSF1 directly promotes the expression of the tight junction protein occludin, ensuring a secure seal and integrity of the intestinal epithelial barrier. This occurs through direct binding to its motif in the occludin promoter region.[102] Consequently, activation of HSF1 during thermal stress within the fever-like range serves as a protective measure against heat-induced disruption of the intestinal tight junction barrier. Conversely, compromised HSF1 activity in T2DM contributes to gut-derived endotoxemia.

Strategies aimed at elevating intestinal HSR and HSP production have been proposed as therapeutic interventions against gastrointestinal toxicity.[103] Alterations in the normal gut microbiota influence mucosal HSP72 expression and may render the organism more susceptible to harmful agents, such as *Clostridium difficile* toxin A.[104] Perturbations in microbiota composition or immune status can contribute to pathogenic processes causing localized intestinal injury.[105,106] Notably, an increase in HSP70 within intestinal barrier cells has demonstrated protective effects in cases of colitis.[107] Additionally, HSP70 polymorphisms appear to influence the severity of intestinal barrier disruptions.[108] Furthermore, small HSPs, such as HSP27, play a role in the cytoskeletal activity of intestinal epithelial cells, crucial for preserving their integrity.[109,110]

Exercise stands as a cost-effective means to combat chronic inflammatory diseases, as extensively documented.[24,25] Notably, exercise also exerts a profound influence on the host-gut microbiota axis.[111] A study involving overweight adolescents who underwent a 10-week program of low-calorie intake and increased physical activity demonstrated beneficial effects on gut microbiota composition. This intervention reduced Firmicutes genera while elevating Bacteroidetes.[111] Exercise alone can impact gut microbiota composition, although taking into account its influence on gut motility and transit time.[112]

The intertwined relationship among diet, metabolism, and exercise has become increasingly apparent, with well-controlled studies on elite athletes shedding light on this synergy (see, for instance,[113] for review). Within this context, whey protein (WP) supplements have gained prominence. Renowned for their postexercise recovery and muscle hypertrophy benefits, WP and whey protein hydrolysates (WPH) have the potential to influence gut microbiota composition and, by extension, lipid metabolism. This notion is supported by metagenomic analyses in relevant samples.[113] Furthermore, WPH is emerging as a novel antidiabetic agent, exhibiting favorable effects on glycemia in animal models and humans. It has been demonstrated to enhance blood glucose clearance, reduce hyperinsulinemia, and restore pancreatic islet insulin secretion in response to glucose in ob/ob mice.[114]

While there is not a "*one-size-fits-all*" optimal gut microbiota composition, given its substantial interindividual variation,[86] manipulations aimed at promoting a more physiologically balanced bacterial phyla (high diversity of genera) composition in the intestines hold promise for enhancing the HSR capacity in the intestines and, consequently, overall health. As exercise mimics many effects of heat therapy on the body, heat

therapy may similarly influence HSR in intestinal barrier cells. Currently, no study has explored the impact of heat therapy on the gut microbiota of individuals with obesity or diabetes. However, we hypothesize that akin to exercise,[26,27] elevating core temperature may induce favorable adaptations in the gut microbiota, potentially enhancing gut barrier function (possibly through increased HSP70 expression in enterocytes), elevating butyrate production, and mitigating chronic LPS/bacterial infiltration into the bloodstream. Our laboratory is actively investigating the effects of heat therapy on gut microbiota to shed light on this intriguing possibility.

Nutraceutical and pharmacological approaches

Various molecules, obtained from both natural sources and synthetic means, have demonstrated their potential as inducers or co-inducers of the HSR, offering benefits in the context of chronic inflammatory diseases. These compounds have been administered as nutraceutical supplements or pharmacological agents, opening promising avenues for therapeutic intervention. Here, we explore some of these approaches.

Commercial WP and WPH represent a notable example of nutraceutical supplementation that has gained popularity, particularly due to their observed positive influence on gut microbiota, as stated in the previous section. WPH, in particular, has demonstrated intriguing anti-inflammatory properties and the ability to augment the HSR. Research on rats subjected to WPH treatment revealed increased HSP70 expression following a single acute treadmill session.[115] Therefore, WPH effects depend on a previous HSR arming stress (exercise). Similarly, a study involving healthy older individuals reported an elevation in HSP70 and HSF-1 levels in response to a combination of resistance training and WPH.[116]

Notably, camel WPH, despite sharing a similar composition with bovine WPH, exhibits superior antioxidant activities due to its higher content of antioxidant amino acids. Studies involving camel WPH have indicated a reduction in NF-κB activity and associated proinflammatory pathways in lymphocytes and hepatocytes in the context of heat stress-induced damage. Importantly, these effects were accompanied by the maintenance of baseline HSP70 levels in both models.[117,118] Indeed, WPH's beneficial effects can be attributed to its amino acid composition, as the effects observed with WPH supplementation were not replicated when casein or nonhydrolyzed WP were administered. This underscores the potential significance of the amino acid profile in hydrolysates.[115]

Furthermore, research has highlighted the benefits of supplementing with nonessential amino acids in various conditions, including exercise and diabetes. In the context of the HSR signaling, these amino acids appear to play a crucial role, as evidenced by the reduction in HSF1 activity observed during amino acid deprivation.[119] Importantly, studies conducted *in vitro* and *in vivo* have demonstrated that the supplementation of L-glutamine or L-alanyl-L-glutamine dipeptide can enhance HSP70 expression in skeletal muscle, liver, and immune cells and tissues of mice. This effect extends to scenarios such as endotoxemia and contributes to improvements in metabolic status and antioxidant profiles.[120–123] Conversely, exercise, a potent trigger of systemic HSR (second only to fever or heat itself), concurrently serves as a significant supplier of glutamine to the circulation.[124] Of note, exercise is linked to increased gut permeability and elevated endotoxin levels in human subjects, particularly in hot environments. A single bout of exercise induces gut damage and heightened permeability in healthy individuals, with exacerbated damage observed in hot conditions.[125] Conversely, oral glutamine supplementation mitigates this impairment.[126] In the same study, it was observed that glutamine enhances the HS-induced expression of HSP70, HSF1, and occludin in cell cultures of the intestinal epithelial line Caco-2.[126] These findings align with previous research (Rearming the HSR through the manipulation of gut microbiota) indicating that HSF1 directly promotes occludin expression, and the HSR improves the intestinal epithelial barrier, thereby preventing LPS-mediated metabolic endotoxemia.[64,65,76,102]

While several amino acids, including glycine, alanine,[127] arginine,[128] and taurine,[129] have demonstrated the capacity to boost HSP70 expression, glutamine stands out as the primary co-inducer within this category. Importantly, in cellular or animal models lacking HSP induction mechanisms, the protective effects of glutamine are not evident,[130,131] thus reinforcing that this amino acid acts by potentiating an existing HSR.

A proposed mechanism for the enhanced HSR elicited by glutamine involves the hexosamine biosynthetic pathway (HBP),[132] as outlined by Leite *et al.*[23] Glutamine serves as a substrate for HBP,[133,134] which, in turn, triggers HSF-1 *N*-acetylglycosylation, ultimately leading to increased HSP70 expression.[132,135] Consequently, glutamine actually operates as a co-inducer of the HSR, necessitating prior activation of HSF1 before the enhancement of HSF1 activity facilitated by glutamine (a concept elaborated upon in the referenced publication).[3]

Glutamine and its derivatives, known enhancers of the HSR, have demonstrated the capacity to ameliorate metabolic status[121,122] and enhance pancreatic β-cell

function both *in vivo* and *in vitro* by bolstering the HSR biochemical pathway.[123,136–138] Studies have also shown that combining glutamine supplementation with exercise provides cytoprotective benefits by augmenting the HSR in animal models.[120,139] However, there are significant unresolved aspects in this context. Notably, plasma glutamine levels have exhibited a positive correlation with coronary artery disease in both male and premenopausal female individuals.[140] A meta-analysis of 10,083 women further revealed that menopausal status is associated with elevated serum glutamine levels.[141] However, plasma glutamine concentrations may not necessarily reflect the organism's capacity for glutamine utilization and its associated effects.[124,142] Moreover, the mechanistic and clinical implications of these findings remain to be fully elucidated, especially given that both chronological age and menopausal status are independently linked to CVD risk factors.[143] Furthermore, conflicting results have emerged from animal models.[144] Currently, there is a dearth of studies specifically investigating the effects of glutamine supplementation, whether alone or in conjunction with HSR inducers like exercise or heat treatment, on menopausal CVD risk factors or hot flushes.[30]

In addition to the natural regulation of the HSR through methods such as heat treatment, either independently or in conjunction with physical exercise or prebiotics, pharmacological intervention offers an alternative approach. An intriguing example of unexplored and potentially valuable molecules lies in the α,β-unsaturated cyclopentenone prostaglandins (cyPGs) of the A-type (but not other *non*-α,β-unsaturated PGs), with PGA_2 being a prominent representative. cyPGs are highly electrophilic compounds naturally produced during the resolution phase of inflammation and exhibit potent anti-inflammatory and antiproliferative properties. They exert their effects by interrupting the entire NF-κB activation pathway.[145] Notably, cyPGs have demonstrated the capability to entirely reverse atherosclerotic lesions both *in vivo* and *in vitro*.[146] A-type cyPGs also engage in physical interactions with 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting step in cholesterol synthesis.[147] This interaction may contribute to the antiproliferative effects of cyPGs, given the indispensable role of cholesterogenesis (not only cholesterol synthesis) in cell proliferation. Consequently, PGA_2, an inducer of the HSR, holds promise as a novel nonstatin inhibitor of (3-hydroxy-3-methylglutaryl-coenzyme A) reductase with potential therapeutic applications in CVD. However, PGA_2's efficacy in chronic inflammatory conditions beyond atherosclerosis[146,148] remains unexplored.

Pharmacological interventions utilizing hydroxylamine derivatives such as bimoclomol and BGP-15 have emerged as promising strategies for addressing a spectrum of metabolic disorders characterized by inflammation. These disorders encompass diabetes, obesity, and related conditions such as CVD, NAFLD, as well as neuromuscular and neurodegenerative ailments.[149] Much like the observed effects of glutamine, which, upon metabolism through the hexosamine biosynthetic pathway (HBP), extends the activation and transcriptional activity of HSF1,[23] bimoclomol and BGP-15 also serve as HSR co-inducers. This implies that these drugs do not instigate a genuine HSR by themselves but rather necessitate a triggering event, such as exercise, heat exposure, or oxidative stress, to potentiate it.

As anticipated, bimoclomol has been demonstrated to accumulate HSP70 in tissues affected by chronic diseases, including diabetes, heart disease, and kidney dysfunction. Importantly, it has exhibited clinical safety in human trials.[150] Animal studies have further substantiated the effectiveness of bimoclomol as an insulin sensitizer, ameliorating peripheral neuropathy in diabetic rats[151] and affording protection to rat cardiomyocytes from severe heat stress (47 °C for 2 h) *ex vivo* and myocardial infarction *in vivo*, with these effects being contingent on HSP70.[152]

Several natural compounds have demonstrated their ability to induce the HSR, leading to an increase in HSP expression in both *in vivo* and *in vitro* settings. Celastrol, a triterpenoid derived from plant root extracts, exhibits anti-inflammatory properties by inhibiting NF-κB and increasing HSP70 levels in cultured cells.[153] Leucinostatin, a fungal peptide mycotoxin, has been shown to enhance HSP70 expression in stressed cells.[154] Carvacrol, also known as cymophenol, a phenolic monoterpenoid found in plant oils, can co-induce HSP70 expression, both *in vitro* and in models of arthritis.[155,156] Geranylgeranylacetone is another HSP70 inducer that demonstrates protective effects in rat models of ischemia-reperfusion injury.[157] Geranylgeranylacetone has also shown promise in improving glucose tolerance in diabetic monkeys through an increase in HSP70 levels[158] and has been considered for use in liver surgery.[159] Similarly, various nontoxic hydroxylamine derivatives known to act as HSP inducers have been investigated. For example, arimoclomol[160,161] besides bimoclomol[162] has been shown to prolong the activity of HSF1, thereby increasing HSP70 induction.[152]

Among the above-mentioned HSR co-inducers, BGP-15, apart from other cellular effects that confer cytoprotection,[163] stands out as a well-studied compound with excellent tolerability and a promising insulin-sensitizing effect.[164–167] Comparative studies in animal models strongly support its potential application in a range of conditions, including CVD, diabetes, metabolic

syndrome, and muscular dystrophies.[168–171] BGP-15 is currently being investigated as a therapeutic option for metabolic diseases.[149,164,172,173] BGP-15, which is a small molecule co-inducer of HSP72, mimics the beneficial effects seen with genetic overexpression of HSP72 and exercise training. These effects include an increase in mitochondrial area and enhanced insulin sensitivity.[149] The interest in BGP-15 as a potential therapeutic agent for inducing the HSR stemmed from its ability to act as a pharmacological mimic of exercise. During exercise, contracting muscle cells significantly increase the flow of glutamine into the circulation and through the HBP. This heightened HBP flow inhibits glycogen synthase kinase-3β (GSK-3β), which usually hampers HSF1 binding to the HS gene promoters. Additionally, the enhanced HBP flow promotes increased AMPK activity, leading to heightened SIRT1 expression and its binding activity to the HSF-1 promoter of HS genes under circumstances of predominantly proteotoxic stress.[2] These combined effects enhance the HSR.[23]

While exercise remains the most potent physiological inducer of the HSR, comparable only to fever, BGP-15, as an HSR co-inducer, not only reduces the production of reactive oxygen species but also possesses the ability to remodel cholesterol-rich membrane domains. Furthermore, it can block the activity of JNKs through direct binding.[173]

In summary, a myriad of molecules, ranging from amino acids to dietary compounds and pharmacological agents, have demonstrated their potential to co-induce the HSR, offering promising avenues for therapeutic intervention in chronic inflammatory diseases and metabolic disorders. Further research is needed to unravel the intricacies of these approaches and translate them into effective clinical strategies.

The integrity of the HSR can be assessed by the Heck index and whole blood heat challenge tests

HSP70 members present some peculiarities regarding their site of responses, stressful situations to those each member may be recruited, and compartmentalization of the products.[174] Elevated iHSP70 expression is closely related to insulin signaling.[31] Insulin itself is a signal for increased expression of iHSP[70175] and regulation of its induction.[176] The intracellular location of HSP70 is related to the preservation and improvement of insulin sensitivity in both skeletal muscle and vascular endothelium.[44,177] Consequently, HSP70 ($HSP72^{-/-}$) knockout mice show poor insulin signaling,[172,178] while transgenic mice overexpressing HSP70 ($HSP72^{+/+}$) present improvement in insulin signal.[149,172] Strikingly, when insulin downstream pathways are turned on, they inhibit the activation of HSF1. There is also a coupling between reduced insulin levels (e.g., restricted nutrient intake) and increased lifespan in a variety of animals, ranging from worms and flies to mice.[179] Actually, we presume that, maybe, HSF1 suppression by insulin is also involved in insulin-mediated insulin resistance[180] and perpetuation of low-grade inflammation in obesity and T2DM! On the other hand, this metabolic behavior is intertwined with the caloristasis equilibrium.[2]

The release of the same HSP70 into the plasma (eHSP70), on the other hand, acts as a danger signal, potentially stimulating the innate and adaptive immune system[181–183] and works as an active mediator of inflammatory pathways, including *via* TLR4 priming.[184] Furthermore, eHSP70 (e.g., eHSP72) has been associated with the danger-associated molecular pattern in activating caspase-1[185] while it has been suggested that a major part of eHSP70 content in the blood is released by circulating immune cells.[186,187] *Acutely*, eHSP70 can be an important defense signal for the maintenance of homeostasis in stressful conditions, being also implicated in motoneuron protection[21] and even in providing anti-inflammatory resources.[188] *Chronic exposure* to high plasma levels of eHSP70, however, is firmly accepted to be deleterious to an array of tissues (including the pancreas) from obese patients and those bearing T2DM, CVD, NAFLD, and menopause-associated metabolic imbalances.[21,30,39,40,189]

The ability to produce and release appropriate amounts of eHSP70 is also associated with the anti-inflammatory HSR,[27,188,190] while the adequate balance between eHSP70 and iHSP70 is now assumed to be directly correlated to the immunoinflammatory status of individuals.[187,191–194] Indeed, increased eHSP70-to-iHSP70 ratios are elevated in different acute and chronic challenges to health[187] but are virtually always associated with low-grade chronic inflammation thus permitting the use of these ratios to follow up patients and laboratory animals.[25] eHSP70 is also linked to arterial hypertension.[195] Besides, elevated eHSP70-to-iHSP70 ratios were found to disrupt vascular responses to calcium and to activate the TLR4/MD2 complex in type 1 diabetes mellitus (T1DM), as reported by De Oliveira and colleagues.[194] This can be partially explained by the rise of a pro-inflammatory cytokine (eHSP70) that mobilizes Ca^{2+} in vascular cells and the reduction of a powerful anti-inflammatory (iHSP70), thus leading to vascular cell dysregulation. Additionally, iHSP70 was found to regulate Ca^{2+} mobilization participating in the contraction of vascular smooth muscle.[194] On the other hand, intracellular Ca^{2+} mobilization within the cytoplasm is a

powerful inducer of HSF1 activation and HSR.[15] Finally, eHSP70 present in the plasma of T1DM rats increases α-adrenergic-induced contraction of aorta rings in a TLR4/MD2-dependent way, collaborating to disturb vascular reactivity in T1DM.[194]

Chronically, the elevation of plasma HSP70 (eHSP72) correlates with tumor necrosis factor-α increase, insulin resistance, and pancreatic β-cell failure.[40] As expected, there is also a negative correlation of eHSP70 with insulin levels and HOMA-IR in overweight young men.[196] Increased eHSP72 levels are associated with sarcopenia[197] and duration of T2DM.[198] On the other hand, conditions that stimulate the tonus of the sympathetic nervous system (as probably occurring during saunas and hot tubs) can augment HSP70 release from immune cells.[24,25] Additionally, adrenergic sympathetic activation occurs following overfeeding.[199] Therefore, in states of continuous surplus energy imbalance,[200,201] such as in obesity, adrenergic stimulus to many tissues may take place. Contrariwise, adipose tissue itself acts as an endocrine organ releasing adipocytokines (e.g. leptin, resistin, adiponectin) that, along with insulin resistance-derived hyperinsulinemia, increase sympathetic activity *via* signaling at the central level, that is, in the nucleus tractus solitarius.[202] Adrenergic stimulus can increase the expression and release of HSP70 by circulating immune cells.[203] Together with the fact that immune cells are reputed to be the main exporters of eHSP70 in stressful situations, measuring the release of HSP70 by this "circulating tissue" became a good tool to assess organismal capacity to activate the HSR under stressful conditions.

The antagonistic relation between intra and eHSP70 signals can be used to assess the inflammatory profile. PBMCs are widely used to access HSP70 production in laboratory animals and humans,[190,204–206] because the expression of HSP70 in PBMC is inversely correlated with the degree of proinflammatory status in tissues from chronic disease patients.[187,190,207] As PBMC evaluation represents a less invasive method that perfectly matches the muscle content of HSP[70,208] we have started to use this approach as a representative of bodily HSP70 intracellular content.

This approach is possible because alterations in proinflammatory parameters are directly correlated with an increased eHSP70-to-iHSP70 ratio, so such ratio can be used as a predictor of the proinflammatory or anti-inflammatory response and to the development of insulin resistance, even before alterations in classical parameters, such as HbA1c, can be noticed.[24,25,209] As previously mentioned, the rationale behind this can be partly explained by the increase in the proinflammatory cytokine eHSP70 and the decrease in the potent anti-inflammatory iHSP70 that correlates with eHSP70-to-iHSP70 ratio. Furthermore, our laboratory's studies have provided clear evidence to support that this postulation is correct.[187] Therefore, taking PBMC and plasmas from patients or laboratory animals allows for the assessment of the time course of the evolution of plasma-to-leukocyte ratios with ease. These ratios can be obtained by a simple division between the absolute values of eHSP70 and iHSP70 in different times or situations, irrespective of the method used to assess these HSP70 contents.

Unlike simply estimating the evolution of eHSP70/iHSP70 ratios, which gives a static picture in different situations, the eHSP70-to-iHSP70 ratio *index* can also be tracked by the Heck index or H-index of organismal HSP70 status. Heck index consists of the comparison between the eHSP70/iHSP70 ratio in a situation with that of the eHSP70/iHSP70 ratio in a different condition, but using the *ratio of eHSP70/iHSP70 ratios*, instead of merely accompanying the linear progression of the eHSP70/iHSP70 ratio.

Heck index has been recently identified as a novel and comprehensive index of an individual's immunoinflammatory status. Similarly to that described above for simple eHSP70/iHSP70 ratios, the Heck index can also be taken by evaluating HSP70 contents in PBMC and plasma, but comparing *the ratio of ratios*. This is possible because eHSP70/iHSP70 ratios between *plasma and PBMC* reflect eHSP70/iHSP70 ratios between *plasma and (metabolic) tissues*.[24,25,181,187,191,207,209–216]

The reasoning for this is that higher eHSP70 levels signify an increase in inflammatory signals, as eHSP70 is inherently proinflammatory. Conversely, cells that respond to stressful stimuli by enhancing intracellular iHSP70 levels are more likely to be in a state of anti-inflammation or equilibrated HSR. To calculate the Heck index, one initially takes $R_c = (eHSP70)_c/(iHSP70)_c$ as the HSP70 ratio in a baseline (control) situation and $R_j = (eHSP70)_j/(iHSP70)_j$ as the HSP70 ratio in any other situation "j," irrespective of the techniques used to measure each eHSP70 and iHSP70. Heck index can then be calculated as the quotient of any R_j by the control R_c, which is considered to be unity ($R_c = 1$) and normalizes all other results in "j" situations allowing for easy comparisons between different conditions. Hence, the Heck index = R_j/R_c and can be used to compare any stressful situation "j" with the assumed control situation. This index can be used to estimate the immunoinflammatory status of an individual (or groups of individuals) in a variety of situations, including immune responses, diabetes, and the immunological impacts of exercise.

As previously shown,[25,187] assuming a Heck index of 1 ($R_c = 1$) for the controls (resting, unstimulated),

exercise can cause a shift in Heck indices of up to approximately 5, which is accompanied by an increase in inflammatory markers and cell proliferation. A Heck index value >5 indicates an exacerbated proinflammatory condition. Conversely, a Heck index value between 1 and 5 suggests a predominantly equilibrated HSR while values <1 indicate an anti-inflammatory status (for more details, see supplemental *Table S2* in Ref.[187]).

Heck index allows for the follow-up of the temporal evolution of immunoinflammatory status in patients and laboratory animals, that is, the occurrence of a background inflammation. High basal Heck indices have been associated with high HOMA-IR values,[39,40] visceral obesity, and insulin resistance,[211] as well as elevated levels of ultra-sensitive C-reactive protein.[217] Heck index is higher in streptozotocin-treated diabetic rats and correlates with vascular abnormalities observed in the animals[194] as well. Consequently, changes in the Heck index have emerged as a potentially novel biomarker for low-grade inflammation and a highly sensitive indicator of an individual's inflammatory status permitting an initial evaluation of the HSR in patients and laboratory animals. As such, maintaining an appropriate balance between extracellular and iHSP70 is now believed to be directly correlated with an individual's immunoinflammatory status.[191–194]

Although the Heck index is especially useful for giving initial clues about the inflammatory status of an individual, it does not furnish the capacity to trigger a pronounced HSR under stress. To supplement the Heck index, we developed a straightforward technique to evaluate HSR status in rodents and humans, using short-term heat challenges of whole-blood samples under different conditions. This method allows the monitoring of HSR integrity capacity,[27] particularly because the expression of HSR components in PBMC is inversely correlated with the degree of proinflammatory "tonus" in chronic disease tissues.[13,190,207] Notably, PBMC incubated at various temperatures for 2 h exhibit an iHSP70 peak at 42 °C, with lymphocytes as the primary producers under this condition.[186] However, after just 1 h of incubation at 40 °C or 43 °C, a significant increase in iHSP70 is observed only at the higher thermal stress level.[206] Similar results are observed upon incubations of circulating monocytes at 41 °C or 42 °C (Schöler *et al.* manuscript in preparation).

Basal iHSP70 contents and thermal stress sensitivity in human PBMC and polymorphonuclear leukocytes differ depending on the cell clusters.[218] Monocytes exhibit a more robust response between 39 °C and 41 °C after a 2-h incubation period, while lymphocytes respond better at 42 °C within the same duration.[219] At temperatures up to 41 °C, lymphocytes and polymorphonuclear leukocytes show only a modest increase in iHSP70, whereas monocytes display a strong induction at 39 °C, with iHSP70 expression at 41 °C being 10-fold higher than in control monocytes at 37 °C.[219] In healthy volunteers who had been exposed to a hot water bath to induce whole-body hyperthermia in a fever-like range (39 °C), iHSP70 induction was observed in all leukocytes, with cell type-specific variations comparable to those observed *in vitro*, albeit less pronounced.[219] Although there are disagreements regarding the temperatures and incubation times used for specific cell types in the literature, our current studies indicate that using a 2-h incubation at 42 °C is optimal for testing iHSP70 production.

To assess organismal HSR under different clinical conditions, we employ a whole-blood HS challenge *ex vivo*. Three approaches are in use in our laboratory, all involving 2-h incubations of whole blood at 42 °C, with control samples kept for the same period at 37 °C. In *protocol #1*, extracellular eHSP70 is promptly measured after a 2-h thermal (or control) challenge,[190] followed by the evaluation of iHSP70 in the PBMC fraction (e.g., Ficoll-Hypaque) after 6 h of rest at 37 °C in 5% CO_2 atmosphere.[220]

In *protocol #2*, we compare both HSP70 in PBMC (intracellular, iHSP70) and supernatant HSP70 (extracellular, eHSP70), after 6 h from the beginning of the 2-h incubation period.[216] We have used the same protocol to compare the HSR in the human ovarian cortex obtained by closed metal container vitrification or the slow-freezing technique of cryopreservation.[221]

Although we believe that the protocols explained above satisfactorily permit evaluation of organismal HSR (see details in Table 2), they have some limitations under specific conditions. A considerable sample volume (at least 400 μL) is required for the test, which for human samples is perfect but is limiting when studying small animals (e.g., mice). Also, the use of gradient-separating products, such as Histopaque or Ficoll-Hypaque (protocol #1 and protocol #2), makes the method more complex and expensive. This is why we set up a simpler and straightforward technique to assess the status of the HSR in rodents and humans by using the short-term heat challenge of whole-blood samples, without requiring prior or post-incubation cell separation. Moreover, changing the culture medium after the heat challenge does not affect the results (Schroeder *et al.* manuscript in preparation). Therefore, *protocol #3* involves diluting whole-blood samples and incubating them at 42 °C (or 37 °C for controls) for 2 h. The samples are then incubated for an additional 6 h at 37 °C, allowing for the accumulation of inducible HSP70 (HSP72), a marker of HSR capacity. By using this

Table 2
Characteristics of current HSR assessment approaches

Technique	Method	Characteristics	Interpretation
Basal measurement	eHSP70 by ELISA kit	Direct measurement with absolute values to compare	Reference values are not known
	iHSP70 by ELISA kit, WB, FCM, IHC	Possible visualization of cellular compartmentalization of the iHSP70	
	Heck index (eHSP70 to iHSP70 ratio index)	Allow quantitation of the ratios between intra and extracellular HSP70 forms as pro/anti-inflammatory markers	$R < 1$ = anti-inflammatory status; $1 \leq R \leq 5$ equilibrated immunoinflammatory surveillance status; $R > 5$ chronic proinflammatory status
Heat shock challenge	Protocol #1 Whole Blood (isolation *after* test)	Provides different combinations and evaluates the overall stimulation of cells	↑HSP70 = HSR preserved
	Protocol #2 Whole Blood (isolation *before* incubation)	Isolation responses from different cell types	
	Protocol #3 Whole Blood (*without* PBMC isolation)	Easy to perform, cost-effective	

Abbreviations used: eHSP70, extracellular HSP70; FCM, flow cytometry; HSP70, 70 kDa family of heat shock proteins; HSR, heat shock response; iHSP70, intracellular HSP70; IHC, immunohistochemistry; PBMC, peripheral blood mononuclear cells; WB, Western blotting.

technique, it is possible to establish the timeline of HSR suppression in animal models of high-fat diet-induced obesity, based on the differences in iHSP70 between the two test temperatures (ΔHSP70). The ΔHSP70 demonstrates a 5-parameter logistic correlation with fasting glycemia (negative), fast insulinemia (negative), HOMA-IR, negative, and Quantitative Insulin Sensitivity Check Index (positive) (Schroeder *et al.* manuscript in preparation). The illustration of *protocol #3* can be found in Figure 1.

Blood samples collected in heparinized tubes are subjected to dispersion and then diluted in a 1:10 ratio with an appropriate culture medium. The resulting diluted blood samples are incubated in a temperature-controlled water bath at 42.00 ± 0.01 °C for a period of

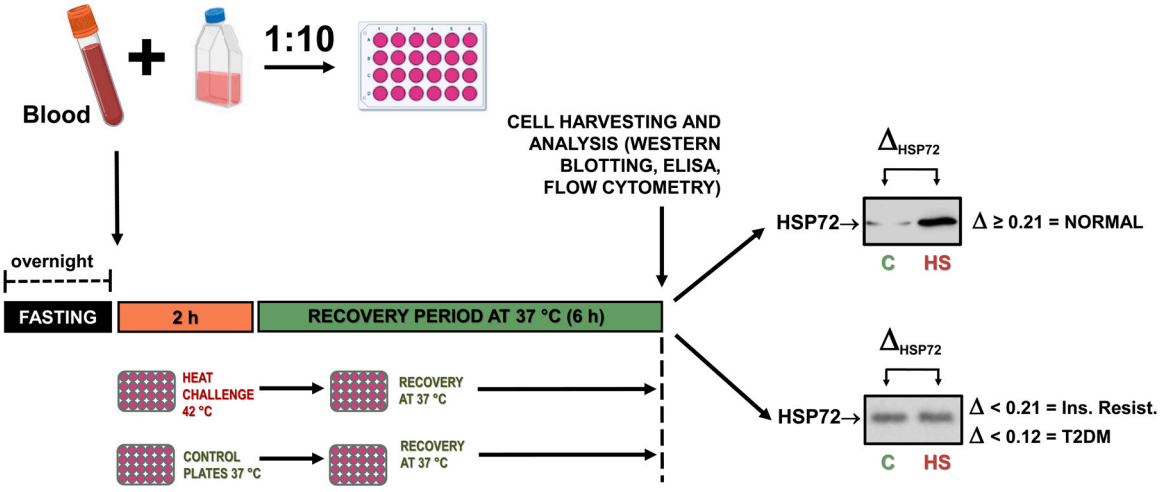

Fig. 1 Whole-blood heat shock challenge *ex vivo* to assess organismal heat shock response. Heparinized blood samples are collected from overnight-fasted individuals and diluted 1:10 with culture medium. Diluted blood samples are, then, incubated in a temperature-controlled water bath at 42.00 ± 0.01 °C for 2 h. Parallel control preparations are to be maintained in another water bath at 37 °C for the same time period. After incubations, cells from both groups are incubated for additional 6 h at 37 °C to allow for a robust accumulation of the inducible forms of HSP70 (HSP72), which serves as a marker of the HSR capacity. Total experimental time, since blood harvesting to the end of incubations, is 8 h. HSP70 accumulation can be assessed by Western blotting, ELISA, and flow cytometry. The *difference* (Δ) between HSP72 expressed after 42 °C compared with that after 37 °C is consistent with IR and T2DM. This illustration was prepared by using free icons from Biorender.com (available at https://app.biorender.com/). Abbreviations used: HS, heat shock; HSP70, 70 kDa family of heat shock proteins; HSR, heat shock response; T2DM, type 2 diabetes mellitus.

2 h. Parallel control preparations are maintained in another water bath at 37 °C for the same duration of time. Following the water bath incubations, cells from both the control and heat-treated groups are incubated for an additional 6 h at 37 °C to allow for the significant accumulation of the inducible forms of HSP70 (HSP72), which serve as markers of the HSR capacity. After the incubation period, cells can be processed for iHSP70 analysis using various techniques, including EIA kits, Western blotting, flow cytometry, or immunofluorescence. This technique is particularly useful when there is no significant difference in basal expressions of iHSP70 between control and test groups, such as in cases of diabetes, obesity, or CVD. In our experience, evaluation of the HSR by *ex vivo* whole-blood HSR protocol #3 is sensitive enough to detect insulin resistance in mice from week to week, even when glucose tolerance tests and basal glycemia are normal.

Concluding remarks

In summary, inflammation, a fundamental physiological response, has evolved as a crucial defense mechanism against microbial threats and tissue damage. However, in our modern Western lifestyle characterized by sedentary habits and excessive calorie consumption, the body's natural mechanisms for resolving inflammation, notably the HSR, become jeopardized. The relentless strain placed on adipose tissue's ER leads to chronic ER stress and the subsequent UPR. Unfortunately, the UPR possesses an inflammatory arm that propagates inflammatory signals throughout the body. This persistent activation of UPR pathways eventually triggers the continuous activation of the NLRP3 inflammasome, initially in adipose tissue, and then affecting the resolution of inflammation by the HSR in multiple tissues beyond just adipose tissue.

This domino effect leads to the establishment of chronic low-grade inflammation, a hallmark of various degenerative conditions including insulin resistance, obesity, T2DM, NFLD, CVDs, and neurodegenerative diseases. Remarkably, strategies do exist to re-establish the HSR, even in the face of persistent noxious stimuli of our Western lifestyle. Heat-based interventions, such as fever and thermotherapy, as well as exercise, operate through distinct mechanisms to dismantle NLRP3 inflammasome activation and reactivate the HSR. This offers hope for alleviating chronic inflammatory conditions.

In this manuscript, we have explored current physiological, pharmacological, and nutraceutical approaches designed to reawaken the HSR in chronic inflammatory conditions. Additionally, we have highlighted clinical tools that can evaluate HSP70 status, offering valuable insights into the HSR's progression or suppression in both patients and experimental animals. Overall, understanding and harnessing the power of the HSR holds promise for helping to combat the widespread burden of chronic inflammatory diseases in our society.

"Quæ medicamenta non sanat æ ferrum sanat. Quæ ferrum non sanat æ ignis sanat. Quæ vero ignis non sanat æ insanabilia existimare oportet.

That which drugs fail to cure the scalpel can cure. That which the scalpel fails to cure heat can cure. If heat cannot cure, it must be determined to be incurable."

(Aphorisms of Hippocrates, by Elias Marks, from the Latin version of Verhoofd, Collins & Co. New York, 1817).

Funding and support This work has been supported by the State of Rio Grande do Sul Foundation for Research Support (FAPERGS/Decit/SCTIE/MS/CNPq/SESRS n. 03/2017-PPSUS #17/2551-0001424-3 to MK and #19/2551-0001713-8 to PIHB) and the Brazilian National Council for Scientific and Technological Development (CNPq, process #303853/2017-4 to PIHBJ, process #307926/2022-2 to TGH and process #302959/2020-3 to MK). Financial support from CAPES (Coordination of Superior Level Staff Improvement) is also acknowledged.

Author contribution HTS and PIHB conceptualized the paper while HTS prepared the first draft. All the authors were involved in co-writing this work. PIHB prepared the figures and supervised the finalization of the manuscript. All the authors have read and agreed to the submitted and published versions of the manuscript.

Data availability statement Data will be made available on request.

Declarations of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. On behalf of all the authors, Paulo Ivo Homem de Bittencourt declares that they have no potential conflict of interest related to the present manuscript, and no competing interests such as consultancies, financial involvement, patent ownership, etc. in relation to the work described. CNPq, FAPERGS, and CAPES (the funding organisms) had no involvement in the propositions presented in this manuscript.

Acknowledgments The authors are indebted to Dr. Maria Inês Lavina Rodrigues for her invaluable technical support during the experiments that support many of the findings described herein. The authors apologize for not including many important primary studies in their text due to space constraints. Their goal was to ensure a smooth flow of information throughout the text.

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Capítulo IV

Título: Progression of insulin resistance revealed by impaired organismal anti-inflammatory heat shock response during ex vivo whole-blood heat challenge

Periódico: Journal Archives of Physiology and Biochemistry

Situação: Submetido (conforme anexo I)

Capítulo V

Título: Chronic whole-body heat treatment in obese insulin-resistant C57BL/6J mice

Periódico: Journal Archives of Physiology and Biochemistry

Situação: Aceito para publicação

DISCUSSÃO GERAL

Esta tese descreveu o histórico do estudo da resposta de choque térmico na proteostase e metabolismo energético; o papel da resposta de choque térmico na resolução da inflamação e no desenvolvimento de doenças crônico-inflamatórias; bem como, métodos de indução e aferição da resposta de choque térmico em três revisões narrativas que compões os capítulos I, II e III. Desta forma, conseguimos demonstrar a interação entre a manutenção da proteostase (a partir da HSR), inflamação e as vias de controle do metabolismo energético. Peças importantes para o entendimento do funcionamento da caloristase, conceito que contribui para o melhor entendimento do desenvolvimento de doenças como obesidade, diabetes, doenças cardiovasculares e neurodegenerativas. Exploramos, também aqui, as atuais abordagens fisiológicas, farmacológicas e nutracêuticas de estímulo da HSR, visando o tratamento de condições inflamatórias crônicas. Além disso, destacamos análises que podem avaliar o status da capacidade do organismo de produzir a HSP70, oferecendo importantes ferramentas de avaliação da condição da HSR em pacientes e animais experimentais.

Na sequência, visando aplicar e ampliar o conhecimento a cerca desses conceitos, realizamos os dois trabalhos experimentais presentes nos capítulos IV e V. O primeiro buscando o acompanhamento do desenvolvimento da obesidade e do perfil de sensibilidade à insulina relacionado à capacidade de resposta ao choque térmico. E o segundo investigando os efeitos da terapia hipertérmica em animais obesos resistentes à insulina nos parâmetros da HSR.

O modelo animal utilizado apresentou falha na capacidade de indução da HSR após a 8ª semana nos animais NC e 1ª semana nos HFD, a despeito da ausência de variação do conteúdo basal de HSP70 intracelular nestes animais. Isto é coerente com o resultado da correlação da variação do conteúdo de HSP70 e o tempo de tratamento dos animais, que demonstraram a antecipação da perda da capacidade de gerar a HSR de 8 semanas do NC para 3 semanas nos animais HFD. O efeito do HT foi testado com quatro e oito sessões, e se mostrou capaz de reduzir o ganho de peso nos animais tratados, melhorando a glicemia de jejum, embora não tenha alterado a resposta no

teste de tolerância à glicose. Em relação à HSR, o tratamento aumentou a presença da forma induzível da HSP70 (HSP72) no gastrocnêmio dos animais.

Em ambos os estudos, foram utilizados nosso teste proposto para medir a capacidade de gerar a HSR nas células imunológicas do sangue. As células mononucleadas de sangue periférico são usadas largamente para acessar a produção da HSP70 em animais e humanos (de Lemos Muller *et al.*, 2018; Lancaster; Febbraio, 2005; Njemini *et al.*, 2002; Rao; Watson; Jones, 1999), pois representa um método menos invasivo e que se correlaciona com o conteúdo muscular de HSP70 (Tuttle *et al.*, 2017). A utilização do desafio térmico nas células imunológicas periféricas do sangue para o acompanhamento da integridade da capacidade da HSR tem sido proposta como um método complementar (de Lemos Muller *et al.*, 2019). E as análises do imunoconteúdo de HSP70 basal e sua resposta ao desafio térmico, realizada em diferentes momentos do desenvolvimento da obesidade e resistência à insulina, mostraram que, de fato, o desafio térmico parece ser uma abordagem de testagem mais sensível, indicando o desequilíbrio na capacidade de armar a resposta ao choque térmico antes de alterar os valores do conteúdo basal. Foi mostrado que o desafio térmico de sangue total pode ser utilizado em fêmeas e machos, desde que em jejum. Podendo ser interpretado em faixas de variação do conteúdo da HSP70 (sua expressão quando submetido a 42 °C menos sua expressão mantida a 37° C). Essas faixas se correlacionaram com a glicose em jejum, HOMA IR e índice quantitativo de verificação da sensibilidade à insulina (QUICKI), demonstrando ser uma importante ferramenta que possibilita o uso de valores preditivos de resistência à insulina. Desta forma, seria interessante a aplicação destas técnicas minimamente invasivas para os estudos utilizando modelos animais, tornando-os translacionais e possibilitando a correlação entre os achados nos tecido metabólicos (e.g., fígado, tecido adiposo, músculos) desses modelos e a evolução das doenças inflamatórias, como proposto no capítulo V.

Com relação ao acompanhamento temporal da IR, parece haver uma adaptação dos animais à dieta após uma semana de tratamento com HFD. Em trabalho anterior de nosso grupo, que acompanhou animais por 10, 14, 18 e 22 semanas de dieta NC ou HFD, da mesma forma que o presente trabalho, foi

observado que os animais tratados com dieta padrão e hiperlipídica apresentaram resultados semelhantes em glicemia, insulinemia e testes de tolerância a insulina e glicose (Schroeder, 2020), resultado que foi reproduzido no presente trabalho. A partir da observação dos resultados citados anteriormente, propomos a adição do acompanhamento por tempos menores (e.g.: 0, 1, 4 e 8 semanas). E a adição dos novos grupos, juntamente com o resultado do desafio térmico, trouxe-nos a perspectiva de que há uma progressiva piora dos animais NC, conforme já comentado, embora, aparentemente, o desequilíbrio seja antecipado pela administração da HFD. Em humanos e primatas, já foi mostrado que a expressão de HSP72 é diminuída no músculo esquelético de pacientes resistentes à insulina e diabéticos tipo 2 (Chung *et al.* 2008; de Matos *et al.*, 2014; Kavanagh *et al.*, 2012; Rodrigues-Krause *et al.*, 2012). Porém, esta relação não parece ser sempre reproduzida em modelos com roedores. No capítulo V, mostrou-se que o HFD por 18 e 22 semanas não foi capaz de alterar a expressão de HSP70. De maneira semelhante aos nossos resultados, em outros trabalhos, com ratos e camundongos, o tratamento com HFD por 6,12, 16 ou 28 semanas não foi capaz de alterar a expressão da HSP70 no músculo dos animais (Gupte *et al.*, 2009a; Gupte *et al.*, 2009b; Bock *et al.*, 2016; Bittencourt *et al.*, 2020). Talvez o tempo de duração da resistência à insulina desses modelos não seja suficiente para provocar alteração na HSR tecidual.

No capítulo V, foram observados efeitos benéficos do HT na glicemia e composição corporal dos animais. Embora, surpreendentemente, esses efeitos não foram acompanhados um aumento na expressão de HSP70 nos tecidos metabólicos estudados (eg.: tecido adiposo, músculos e fígado). A associação desses fatores já foi mostrada em trabalhos anteriores. Um estudo em camundongos *knockout* para expressão de HSP72 mostrou que a expressão desta proteína é necessária para a redução da glicose no sangue com tratamento hipertérmico crônico (Von Schulze *et al.*, 2021). E já foi demonstrado que o aumento da expressão de HSP72 no músculo protege os animais do desenvolvimento de resistência à insulina induzida por uma dieta rica em gordura (Henstridge *et al.*, 2014). Resultados de redução significativa nos valores de glicemia de jejum e insulina, bem como do HOMA IR dos

animais após 48h e 72h da última sessão de choque térmico foram relatados na literatura (Chung *et al.*, 2008; Karpe; Tikoo, 2014).

A ausência de ativação do HSR pode indicar que o tempo de tratamento foi insuficiente para o presente modelo. Assim como a frequência de uma vez por semana, amplamente utilizada (Bruxel *et al.*, 2019; Gupte *et al.*, 2009; Von Schulze *et al.*, 2021; Chung *et al.*, 2008), não é suficiente para superar o platô na capacidade de lidar com mais estresse (obesidade e IR) nestes animais. Resultados semelhantes já foram obtidos em um trabalho anterior, de nosso laboratório, com exercício em camundongos obesos (Bittencourt *et al.*, 2020). Por outro lado, a HT reduziu o ganho de peso, melhorou a glicemia de jejum, o que pode estar associado ao aparecimento da isoforma HSP72 no músculo. Foi observado em estudos com humanos obesos, resistentes à insulina ou DMT2 que a HSP70 está reduzida no tecido muscular, e sua expressão inversamente correlacionada com fatores como percentual de gordura corporal (Chung*et al.*, 2008; Henstridge *et al.*, 2010; Rodrigues-Krause *et al.*, 2012; de Matos *et al.*, 2014). Desta forma, o HT apresentou efeito positivo nos animais T22 HFD, que poderia estar ligada a alteração da composição corporal. Pois, considerando a relação da inflamação com a expansão do tecido adiposo (de Lemos Muller *et al.*, 2018; Tomeleri *et al.*, 2016), seria esperado que uma melhora da HSR estivesse ligada a uma redução deste tecido.

Como forças dos trabalhos experimentais, pode-se citar o longo período de tratamento dos animais e seu acompanhamento em oito pontos distintos para a comparação dos seus efeitos. E como principal limitação dos estudos experimentais, temos a utilização da medida do conteúdo de HSP70 intracelular exclusivamente pela técnica de westen blot, que apresenta apenas uma análise semi-quantitativa e pode ser menos sensível do que outras técnicas como citometria e ELISA.

CONCLUSÃO FINAL

Tem-se como principais conclusões da presente tese que o entendimento da interação entre a manutenção da proteostase, inflamação e metabolismo energético, mediada pela resposta de choque térmico, é fundamental para o manejo e propostas de intervenção em doenças como obesidade e diabetes. A avaliação da capacidade do organismo de produzir a HSP70, pela técnica de desafio térmico, se relaciona diretamente com o índice glicêmico dos animais estudados e pode ser uma importante ferramenta diagnóstica.

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ANEXO I – CARTA DE SUBMISSÃO DO ARTIGO 4

De: IARP-peerreview@journals.tandf.co.uk <IARP-peerreview@journals.tandf.co.uk>
Enviada em: 03 April 2024 13:08
Para: pauloivo@ufrgs.br
Assunto: Submission received for Archives of Physiology and Biochemistry (Submission ID: 242515110)

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Dear Paulo Ivo Homem de Bittencourt Jr.,

Thank you for your submission.

Submission ID	**242515110**
Manuscript Title	**Progression of insulin resistance revealed by impaired organismal anti-inflammatory heat shock response during ex vivo whole-blood heat challenge**
Journal	**Archives of Physiology and Biochemistry**

If you made the submission, you can check its progress and make any requested revisions on the Author Portal
Thank you for submitting your work to our journal.
If you have any queries, please get in touch with IARP-peerreview@journals.tandf.co.uk.
Kind Regards,
Archives of Physiology and Biochemistry Editorial Office

ANEXO II – CARTA DE ACEITE DO ARTIGO 5

From:IARP-peerreview@journals.tandf.co.uk
Sent:10-09-2024 8:20 PM
To:pauloivo@ufrgs.br
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Subject: Re: NOTICE: Outstanding information required for your accepted manuscript

249669411.R1 - Chronic whole-body heat treatment in obese insulin-resistant C57BL/6J mice

Dear Paulo Ivo Home de Bittencourt Jr.,

Congratulations on your accepted manuscript in Archives of Physiology and Biochemistry.

I am continuing to process your accepted manuscript (referenced above). To prepare your manuscript for publication, I require the following:

- I require the source files for your MAIN DOCUMENT/FIGURES/SUPPLEMENTARY FILE as we cannot proceed with a PDF for the main text. Please could you email me the source files for your main document, tables and figures to the address above. Any figures should be saved as either .ps, .eps, .tif or .jpeg file types. If you have built your paper in LaTex, please send me all of your individual files in a zipped archive file (.zip or .rar), ensuring that all relevant .sty, .bib, .cl etc. supplementary files are included, so that the manuscript can be correct built.
- Please provide the main manuscript with author details without any highlights, tracked changes, and/or cross-offs.

As I will be unable to proceed without them, I would be grateful if you could provide your files for this submission as soon as possible.

Best regards,

Surya Prabhu (he/him) - Journal Editorial Office

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