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Avaliação de Sistema Purinérgico em Ratos Submetidos a Estresse Crônico - *Interações Farmacológicas e Bioquímicas*

Iraci Lucena da Silva Torres

Orientadora: Profª. Drª. Maria Beatriz Cardoso Ferreira

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AA: ácido araquidônico

ACTH: hormônio adrenocorticotrófico

ADO: adenosina

ADP: adenosina difosfato

AMP: adenosina monofosfato

AMPA: a-amino-3-hidróxi-5-metil-isoxazol-4- ácido propiônico)

ATP: adenosina trifosfato

AVP: argenina-vasopressina

CD39: ecto-apirase

COX-1: cicloxigenase 1

COX-2: cicloxigenase 2

CPA : N^6-ciclopentiladenosine

CRH: hormônio liberador de corticotrofina

DAG: dialcilglicerol

DP: dipiridamol

DPCPX: 1,3-dipropil-8-ciclopentilxantina

E-NPP: ectonucleosídeo pirofosfatase/fosfodiesterase

E-NTPDase: ectonucleosídeo trifosfato difosfato-hidrolase

GABA: ácido γ-aminobutírico

GCs: glicocorticóides

H: hora

HHA: eixo hipotálamo-hipófise-adrenal

IP_3: inositol trifosfato

I.P: intraperitonial

KA: cainato

L: litro

LTP: potenciação de longa duração (*long-term potentiation* em inglês)

Mg/kg: miligramas por quilo de massa corporal

Nadr: noradrenalina

NO: óxido nítrico

NDP: nucleotídeo 5'difosfato

NMDA: N metil D aspartato

NTP: nucleosídeo 5'trifosfato

PC: fosfatidilcolina

PGE_2: prostaglandina E_2

PKC: proteína quinase C

PLC: fosfolipase C

PLA_2: fosfolipase A_2

PIP_2: fosfatidil-inositol 4,5-bifosfato

POMC: pró-ópio-melanocortina

PVN: núcleo paraventricular do hipotálamo

SA: simpático-adrenal

SIA: analgesia induzida por estresse (*stress-induced analgesia* em inglês)

SNC: sistema nervoso central

SSIA: analgesia induzida por estresse por natação (*swim stress-induced analgesia* em inglês)

VIP: peptídeo intestinal vasoativo (*vasoactive intestinal polypeptide em inglês)*

RESUMO

Diferentes efeitos têm sido observados sobre a resposta nociceptiva após a exposição a estresse agudo ou crônico em ratos. A exposição a estresse agudo induz analgesia e a estresse crônico, hiperalgesia. O sistema purinérgico é sabidamente envolvido em mecanismos de nocicepção (ativando ou inibindo). Nessa tese, avaliaram-se a nocicepção em ratos estressados cronicamente por imobilização e alguns parâmetros a ela relacionados. Além disso, avaliou-se a hidrólise de nucleotídeos de adenina em diferente estruturas do SNC (medula espinhal, córtex cerebral, hipotálamo) e soro. Ratos Wistar adultos foram repetidamente estressados por 40 dias, o que induziu hiperalgesia, observada no aparelho de *tail-flick*. A hiperalgesia observada em machos após estresse crônico é dependente de gênero, uma vez que não ocorreu em fêmeas. Nova sessão de estresse agudo foi aplicada após os 40 dias de estresse, observando-se analgesia após a sessão de nado forçado, mas não após imobilização. Avaliou-se também o efeito de agonista de receptor A1 de adenosina, N^6-ciclopentiladenosina (CPA, 3,35 mg/kg, por via i.p.) e de um antagonista de receptor A1 de adenosina, 1,3-dipropil-8-ciclopentilxantina (DPCPX, 0,8 mg/kg, por via i.p.), assim como o efeito de um inibidor de transporte de nucleosídeos, dipiridamol (DP, 5 mg/kg, por via i.p.), na nocicepção de animais controles e cronicamente estressados. O grupo controle apresentou aumento da latência de *tail-flick* após a administração de CPA e DP, mas nenhum efeito foi observado no grupo cronicamente estressado. DPCPX não produziu alteração de nocicepção em quaisquer dos grupos. Os efeitos analgésicos de CPA e DP foram revertidos por DPCPX nos animais controle, indicando envolvimento de adenosina e, mais especificamente, de receptores A1 de adenosina na antinocicepção observada. A ausência de efeito em animais estressados sugere que a sinalização da dor induzida por estresse crônico apresenta uma modulação diferente, envolvendo o sistema adenosinérgico. Uma NTPDase (apirase) hidrolisa ATP e ADP em sinaptossomas de sistemas nervosos central e periférico. Considerando os resultados farmacológicos obtidos com agonistas e antagonistas de receptores de adenosina, investigou-se a hidrólise de nucleotídeos de adenina em medula espinhal de ratos machos e fêmeas estressados crônica e agudamente. Ratos Wistas adultos machos e fêmeas foram submetidos a 1 h/dia a estresse por imobilização por 1 dia (agudo) ou por 40 dias (crônico), foram sacrificados 24 horas após a última sessão de estresse. Atividades ATPásica-ADPásica foram medidas em sinaptossomas de medula espinhal de ratos controles e estressados. A hidrólise do ADP mostrou-se diminuída em 25% nos animais machos cronicamente estressados. Houve também neste grupo aumento na atividade da 5'nucleotidase, enzima que hidrolisa AMP extracelular. Nenhum efeito foi observado em fêmeas cronicamente estressadas ou em estresse agudo tanto em machos quanto em fêmeas. Avaliou-se então a hidrólise de nucleotídeos de adenina em sinaptossomas de duas estruturas cerebrais (córtex frontal e hipotálamo) e soro de ratos machos. Não foi observada qualquer alteração de atividades ATPásica ou ADPásica após estresse crônico nas estruturas cerebrais analisadas. Por outro lado, houve redução de 27% na hidrólise do ADP, sem alteração na atividade ATPásica em soro dos animais cronicamente estressados. É possível que esse efeito represente uma adaptação ao estresse crônico, podendo refletir diferentes funções

de nucleotídeos e/ou enzimas nessas frações. Além disso, também é possível que níveis alterados da atividade ADPásica no soro possa servir como marcador bioquímico de estresse. Aumento da concentração de ADP, sabidamente um indutor de agregação plaquetária, em soro pode sugerir um papel para esse fator na etiologia da arterosclerose produzida por estresse. Investigou-se então o efeito do estresse agudo na hidrólise de nucleotídeos de adenina em soro de ratos, utilizando diferentes tempos de avaliação após a sessão de estresse. Ratos adultos machos foram submetidos a 1 hora de estresse de imobilização e foram mortos após 0, 6, 24 e 48 horas. Houve aumento da hidrólise de ATP e ADP 24 horas após o estresse (58% e 54% respectivamente, quando comparado ao grupo controle). Por outro lado, a hidrólise de AMP aumentou 6 e 24 horas (68% e 94% respectivamente, em comparação ao controle) após o estresse. Esses efeitos podem refletir a presença de mecanismo de proteção ao estresse e as alterações enzimáticas observadas em soro podem ser marcadores bioquímicos de exposição aguda a um estressor.

ABSTRACT

Different effects upon the nociceptive response have been observed with exposure to acute and chronic stress in rats. The acute stress has induced analgesia, and, in the other hand, the chronic stress has induced hiperalgesia. The purinergic system is involved in the nociception mechanisms. In this thesis, we aimed to study nociception in chronically stressed rats and to evaluate some parameters related to nociception in these animals. In addition we evaluated the nucleotides hydrolysis in different fractions (spinal cord, cerebral cortex, hypothalamus and blood serum). Adult Wistar rats were repeatedly submitted to restraint for 40 days, and the nociceptive response was evaluated using the tail-flick test. The effect hiperalgesic observed in the male rats is gender-dependent. Exposuring the animals to a new session of acute stress at the end of 40 days period, the chronically-stressed rats demonstrated analgesia after forced swimming, but not after restraint. We also evaluated the effects of adenosine A1 receptor agonist, N^6-cyclopentyladenosine (CPA, 3.35 mg/kg, i.p.), and adenosine A1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.8 mg/kg, i.p.), as well the effect of nucleosides transport inhibitor, dipyridamole (DP, 5 mg/kg, i.p.), upon nociception in chronically-stressed and control rats. The control group showed increased tail-flick latencies after administration of CPA and DP, but this effect was not observed in the stressed group. DPCPX did not produce analgesic effect in neither groups. The analgesic effect of CPA was reverted by DPCPX and dipyridamole in the control animals. These results indicate the involvement of adenosine and adenosine A1 receptor in the antinociception observed in the control group and suggest that the pain signaling induced by chronic stress presents a different modulation involving the adenosinergic system. No effect was observed in females. There is increasing evidence that both ATP and adenosine can modulate pain. NTPDase (apyrase) hydrolyze extracellular ATP and ADP in synaptosomes from the peripheral and central nervous system. Considering to the pharmacological effects observed before, we investigated the effect of chronic and acute stress on ATPase-ADPase and 5'nucleotidase activities in spinal cord of male and female rats. Adult male and female Wistar rats were submitted to 1 h/day restraint stress for 1 day (acute) or 40 days (chronic) and were sacrificed 24 h later. ATPase-ADPase activities were assayed in the synaptosomal fraction obtained from the spinal cord of control and stressed animals. ADP hydrolysis was decreased 25% in chronically stressed males, while no change was observed on ATPase activity. There was an increase in the 5'nucleotidase activity, an enzyme that hydrolyze extracellular AMP, in the same group. No effect on ADPase, ATPase and 5'nucleotidase activity was observed in chronically stressed females, and after acute stress neither in males or females. In addition, we evaluated the effect of chronic stress on the hydrolysis of adenine nucleotides in two cerebral structures (frontal cortex and hypothalamus) and in the blood serum of male rats. No effect on ADPase or ATPase activities was observed in synaptosomal fraction of any of the cerebral structures analyzed after chronic stress. On the other hand, ADP hydrolysis was decreased 27% in chronically stressed males, while no change was observed on ATPase activity. It is also possible that the effects observed in blood serum may represent an adaptation to chronic stress and may reflect different functions of nucleotides and/or enzymes in these fractions. It is possible that altered

levels of ADPase activity in serum may be a biochemical marker for chronic stress situations. The increased ADP concentration in the serum of chronically stressed animals may suggest the role of this factor in the etiology of atherosclerosis, since ADP is known as platelet aggregation inductor. Thus, we investigated the effect of acute stress on the hydrolysis of adenine nucleotides in rat blood serum. Adult male Wistar rats were submitted to 1 h restraint stress and were sacrificed at 0, 6, 24 and 48 h. Increased ATP and ADP hydrolysis was observed in the blood serum of stressed rats 24 h after the stress session (58% and 54 % respectively when compared to controls). On the other hand, the AMP hydrolysis was increased after 6 h and 24 h (68% and 94% respectively when compared to controls) after stress. These effects may represent a protective mechanism against the effects of stress and the altered activity of soluble enzymes in serum may be a biochemical marker for acute stress situations.

INTRODUÇÃO

1. Estresse

A vida torna-se possível devido à manutenção do organismo em equilíbrio dinâmico constante ou homeostase. Este equilíbrio é freqüentemente alterado por fatores adversos intrínsecos ou extrínsecos, que são os estressores. O estresse é um estado, e o estressor é seu fator desencadeante (causa). O sistema de estresse coordena a resposta adaptativa do organismo a algum tipo de estressor.

Selye (1936) definiu estresse como uma resposta estereotipada, não específica do corpo a alguma alteração. Esta resposta foi chamada de síndrome de adaptação geral e é caracterizada por hipertrofia de adrenal, sangramento gastrintestinal e diminuição da função de órgãos do sistema imune (Selye, 1974). A partir dos trabalhos de Selye (1936), um maior entendimento do mecanismo de estresse tem sido obtido e tornou-se possível identificar diferentes doenças relacionadas a seus mecanismos de adaptação. Especulações de que o estresse pode contribuir para alterações neurodegenerativas e de que o mecanismo de envelhecimento patológico pode estar associado a eventos estressantes são de considerável interesse.

O cérebro recebe impulsos produzidos por vários estressores e envia respostas por meio dos sistemas nervoso, endócrino e imune. Atualmente sabe-se que as respostas adaptativas têm alguma especificidade em relação ao estressor que a produziu, embora esta seja progressivamente perdida quando a intensidade do estressor aumenta. A ativação de sistemas envolvidos com estresse leva a mudanças comportamentais e periféricas que melhoram a habilidade do organismo de manter a homeostase, aumentando a chance de sobreviver. Durante o estresse, a atenção é aumentada e o cérebro focaliza a ameaça. As freqüências cardíaca e respiratória são aceleradas, o catabolismo é aumentado e o fluxo sangüíneo é

redirecionado para prover maior perfusão e combustível para cérebro, coração e músculos (para revisão de estresse, ver Tsigos & Chrousos, 2002).

Alterações no meio ambiente levam, em termos neuroendócrinos, a respostas hormonais que têm como objetivo manter a homeostase do organismo. Eixos hipotalámo-hipófise-adrenal (HHA) e simpático-adrenal (SA) são assim ativados pelos estressores (figura 1). Como imediata resposta, alteram-se a taxa de descarga dos neurônios simpáticos e a secreção hormonal de catecolaminas no sangue. A resposta simpática leva então a aumento de freqüências cardíaca e respiratória e de pressão sangüínea, broncodilatação, dilatação de pupilas, transpiração e palidez. No pico da resposta, fontes fisiológicas de energia são mobilizadas. O estágio mais tardio é caracterizado pela liberação de hormônios como cortisol. Esta etapa final pode funcionar como mecanismo supressivo, reduzindo as respostas orgânicas e re-estabelecendo o balanço fisiológico (Ursin & Olff, 1993). Repercussões patológicas parecem estar associadas com prolongados e sustentados estados de estresse, em que os mecanismos homeostáticos são mais exigidos (Ursin & Olff, 1993).

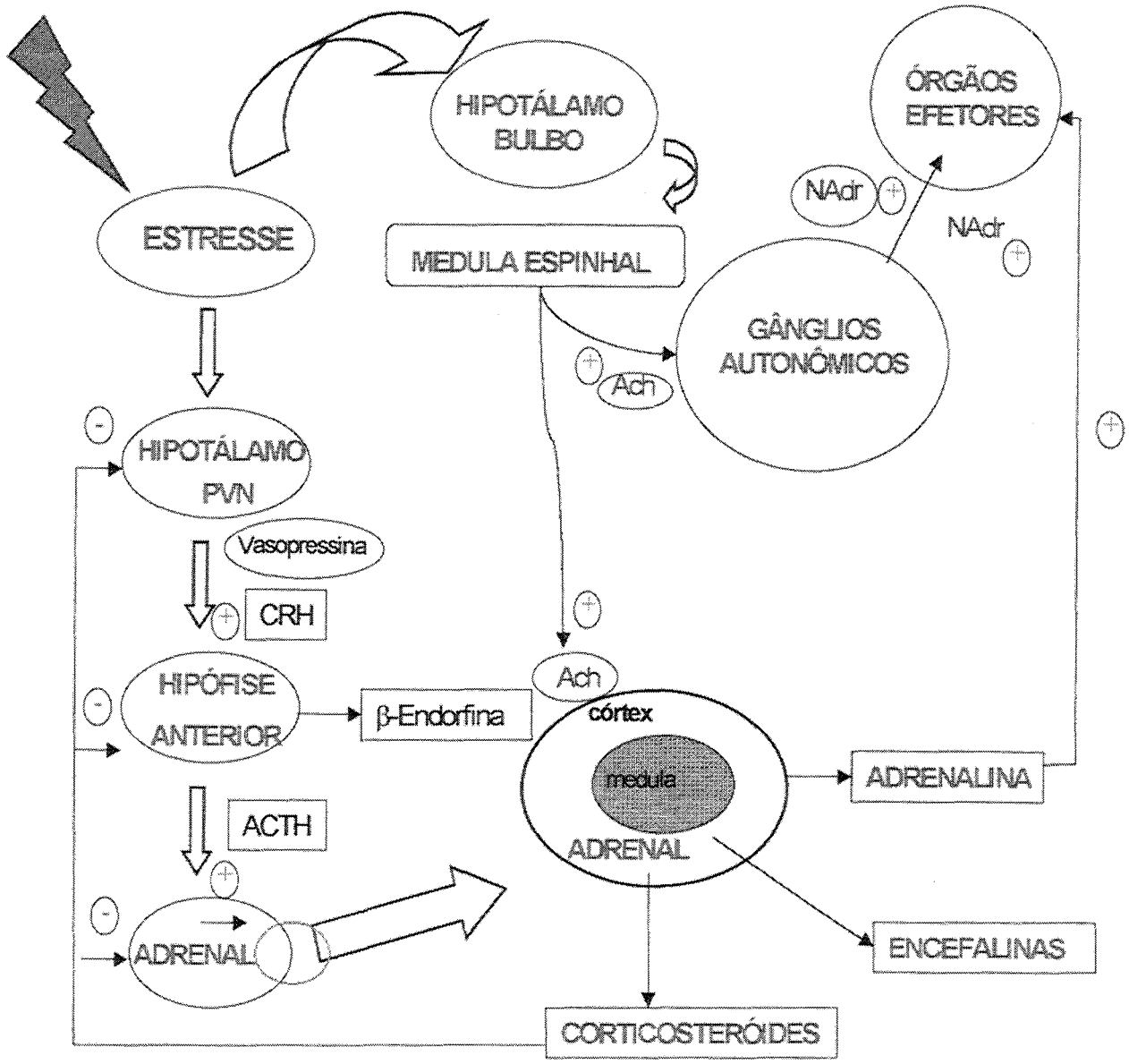

Figura 1: Representação esquemática da ativação de eixos hipotálamo-hipófise-adrenal (HHA) e simpático-adrenal (SA) pelo estresse. PVN= núcleo para-ventricular hipotalâmico; CRH= hormônio liberador de corticotrofina; ACTH= hormônio adrenocorticotrófico.

Várias vias de entrada (*inputs*) relacionadas com o estresse convergem para neurônios do núcleo paraventricular hipotalâmico (PVN – h*ypothalamic paraventricular nucleus*) que sintetizam, entre outros, o hormônio liberador de corticotrofina (CRH- *corticotrophin releasing hormone*) e argenina-vasopressina (AVP) (figura 1). Estes neurônios projetam-se para a eminência média, onde seus produtos são liberados na circulação porta. Esses produtos agem na hipófise anterior e levam à síntese e à liberação de hormônio adrenocorticotrófico (ACTH – *adrenocorticotrophic hormone*) e outros peptídeos derivados de um precursor comum, a pró-ópio-melanocortina (POMC). Vasopresina atua como fator sinérgico junto ao CRH na estimulação da secreção de ACTH. O ACTH, por sua vez, ativa biossíntese e liberação de glicocorticóides (GCs) do córtex da adrenal (corticosterona nos roedores e cortisol nos primatas). Os GCs são os efetores finais do eixo HHA e participam no controle da homeostase do organismo e da resposta ao estresse. Eles têm função-chave na regulação da atividade basal do eixo HHA e na finalização da resposta ao estresse pela ação em centros extra-hipotalâmicos, hipotalâmicos e hipofisários (Tsigos & Chrousos, 2002). A retroalimentação negativa de GCs sobre resposta secretória ao ACTH atua como fator limitante do tempo de exposição dos tecidos a GCs, minimizando assim seus efeitos catabólicos, anti-reprodutivos e imunossupressores.

Esses esteróides possuem atuação extremamente ampla, mediada por receptores especializados que afetam expressão e regulação de genes, resultando em mudanças em vários processos metabólicos. Entre os eventos observados em resposta a GCs, incluem-se, por exemplo, alterações em respostas imunológicas e processos inflamatórios, além da resposta ao estresse (tabela 1). Muitos desses

processos são necessários para adaptação e preparação do organismo para lidar com a situação estressante, incluindo mudanças em forma de obtenção de energia e metabolismo (Cullinan *et al.*, 1995, Akil & Morano, 1995, Schimmer & Parker, 2001).

Tabela 1 - Efeitos de corticosteróides

EFEITOS METABÓLICOS E SOBRE O EQUILÍBRIO HIDROELETROLÍTICO
♦ Menor utilização periférica de glicose
♦ Aumento de gliconeogênese e armazenamento de glicogênio
♦ Menor síntese e maior degradação protéica
♦ Indução de lipólise e redistribuição de gorduras
♦ Retenção de sódio e perda de potássio
♦ Menor absorção gastrintestinal e maior excreção renal de cálcio
ATIVIDADE IMUNOSSUPRESSORA

No sistema nervoso central, os GCs afetam humor e comportamento. Alterações do eixo HHA têm sido identificadas em pessoas depressivas, sugerindo envolvimento de GCs na etiologia de depressão e outras doenças (Young *et al.*, 1991).

A ativação do eixo HHA representa a manifestação primária do estresse, mas deve-se também levar em conta aspectos adicionais da resposta do sistema nervoso e suas potenciais interações para um completo entendimento de seus efeitos. Assim o aumento em conjunto dos níveis plasmáticos de adrenalina e cortisol leva à maior liberação hepática de glicose (Berne & Levy, 2000). A adrenalina liberada ativará vias da glicogenólise hepática, enquanto que o cortisol disponibilizará mais

aminoácidos como substrato para gliconeogênese, aumentando o suprimento de glicose no sangue. Esse combustível deixa o músculo do animal preparado para luta ou fuga (Berne & Levy, 2000). A ativação do sistema nervoso simpático em resposta a um evento estressante causa ainda uma série de repercussões orgânicas descritas na tabela 2 (Hoffman & Taylor, 2001).

Tabela 2 - Efeitos decorrentes da liberação de adrenalina e estimulação de receptores autonômicos. (adaptado de Hoffman & Taylor, 2001)

Sistemas e órgãos	Efeitos Simpáticos	Receptores Simpáticos
CARDIOVASCULAR		
-coração		
▪ freqüência cardíaca	aumento ++	beta-1 e beta-2
▪ força de contração	aumento	beta-1 e beta-2
- arteríolas		
▪ coronárias e		
▪ músculos esqueléticos	constrição +/dilatação++[1,2]	alfa e beta-2
▪ pulmonares	constrição+/dilatação++[1]	alfa-1 e beta-2
▪ vísceras abdominais	constrição+++/dilatação[2]	alfa-1 e beta-2
▪ renais	constrição+++/dilatação+[2]	alfa, beta-1 e beta-2
▪ pele e mucosas	constrição+++	alfa
▪ cerebrais	constrição (leve)	alfa-1
▪ glândulas salivares	constrição+++	alfa
▪ veias (sistêmicas)	constrição++ / dilatação++	alfa e beta-2
RESPIRATÓRIO		
- secreções	redução	alfa-1
	Aumento	beta-2
- musculatura brônquica	relaxamento+	beta-2
OLHO		
- musculatura ocular		
▪ radial da íris	contração (midríase)++	alfa-1
▪ ciliar	relaxamento+	beta-2
▪ glândulas lacrimais	secreção	alfa (predomínio)
PELE		
▪ músculos piloeretores	contração ++	alfa-1
▪ glândulas sudoríparas	secreção localizada +	alfa-1
BAÇO		
▪ cápsula esplênica	contração +++	alfa-1
	Relaxamento +	beta-2

[1]Contração predomina *in situ* devido aos fenômenos auto-reguladores metabólitos.

[2]Acima da variação habitual da concentração de adrenalina circulante, a resposta dos receptores β (vasodilatação) predomina nos vasos sangüíneos do músculo esquelético e fígado, a resposta dos receptores α (vasoconstrição) predomina nos vasos sangüíneos de outras vísceras abdominais.

Sendo assim, a resposta neuroendócrina ao estresse leva a alterações em vários sistemas, como mostrado na tabela 3 (Ferreira, 2003).

Tabela 3 - Resposta neuroendócrina ao estresse. (Ferreira, 2003)

Sistemas	Efeitos observados
Cardiovascular	Aumento de pressão arterial, taquicardia, alterações de débito cardíaco (aumento na maioria dos indivíduos normais, redução em pacientes com comprometimento da função ventricular), aumento do consumo miocárdico de oxigênio.
Respiratório	Aumento do consumo total de oxigênio e da produção de dióxido de carbono, com conseqüente aumento da ventilação por minuto. Maior trabalho respiratório, especialmente em pacientes com doença pulmonar.
Gastrintestinal e geniturinário	Aumento do tônus esfincteriano e redução das motilidades intestinal e urinária (distensão abdominal e retenção urinária). Hipersecreção de ácido gástrico, favorecendo formação de úlcera de estresse. Náusea, vômito, constipação.
Endócrino	Na circulação, ocorre aumento de hormônios catabólicos (adrenalina, cortisol e glucagônio) e redução de hormônios anabólicos (insulina e testosterona). Balanço nitrogenado negativo. Hiperglicemia. Aumento de lipólise. Retenção de sódio e água por aumento de renina, aldosterona, angiotensina e hormônio antidiurético (associado ao aumento de cortisol).

Hematológico	Aumento da adesividade plaquetária, redução da fibrinólise, tendência à hipercoagulabilidade.
Imunológico	Leucocitose com linfopenia e depressão da atividade retículo-endotelial, predispondo a infecções.

Animais cronicamente estressados não têm apresentado o mesmo padrão de comportamento e nem as mesmas respostas neuroquímicas e farmacológicas de animais estressados agudamente (Cancela *et al.*, 1995, Torres *et al.*, 2001a e b). Diferentes sistemas neurotransmissores têm sido sugeridos como tendo função no processo de adaptação ao estresse (Cancela *et al.*, 1995). Assim, exposição repetida a essas situações modifica a resposta comportamental a opióides e a resposta fisiológica à administração de agentes colinérgicos (Filkestein *et al.*, 1985, Disalver *et al.*, 1986, Cancela *et al.*, 1995, Torres *et al.*, 2001b).

Estudos prévios têm mostrado que a resposta do eixo HHA é progressivamente reduzida após exposição repetida ao estressor, fenômeno conhecido como habituação. Esta pode ocorrer após exposição a diferentes formas de estresse crônico repetido, como manipulação, imobilização, sons etc. Depende de vários fatores, entre eles intensidade do estresse e intervalo de tempo entre as sessões. Há também variabilidade individual (Lachuer *et al.*, 1994). A habituação representa mecanismo compensatório que atenua os efeitos deletérios do estresse repetido e tenta manter a homeostase. Esta habilidade para adaptar-se ao estresse repetido e prolongado parece ocorrer em nível de eixos HHA e SA, os dois maiores sistemas envolvidos na resposta ao estresse (Mansi & Drolet, 1997). Esse processo coincide temporalmente com o início de mudanças em sistemas monoaminérgicos. Como nessa situação os sistemas neuroendócrinos não respondem mais da mesma

maneira, tem sido sugerido que regime prévio de estresse crônico poderia modificar respostas comportamentais determinadas pela subseqüente exposição a uma nova situação de estresse (Cancela *et al.*, 1995).

Atenuação da resposta da hipófise ao estresse crônico pode envolver mecanismos que incluem aumento na retroalimentação negativa dos GCs, diminuição da secreção hipotalâmica de CRH, exaustão da capacidade secretória de corticotróficos e diminuição de receptores da hipófise para reguladores de ACTH (Hauger *et al.*, 1988).

2. Dor, nocicepção e hiperalgesia

Dor é fenômeno difícil de ser definido, sendo geralmente apresentado por meio de exemplos de situações dolorosas. A Associação Internacional para o Estudo da Dor a conceitua "como uma experiência sensorial e emocional desagradável, relacionada com lesão tecidual real ou potencial, ou descrita pelo indivíduo em termos deste tipo de dano" (Jacobson & Mariano, 2001). Compreende, portanto, dois componentes – nocicepção e reatividade à dor.

A dor é sempre uma manifestação subjetiva. Em experimentos com animais, fala-se em nocicepção. O termo nocicepção, derivado de *noci* (dano ou injúria em latim), é usado para descrever a resposta neural a estímulos traumáticos ou lesivos. Refere-se à atividade do sistema nervoso aferente induzida por estímulos nocivos, tanto exógenos (mecânicos, químicos, físicos e biológicos) quanto endógenos (inflamação, aumento de peristaltismo, isquemia tecidual). Compreende recepção dos estímulos por estruturas periféricas específicas, condução até o sistema nervoso central, através de vias nervosas sensitivas, e integração da sensação dolorosa em níveis talâmico e cortical (Ferreira & Torres, 2004).

A reação à dor compreende uma série de comportamentos defensivos - desde a retirada reflexa da área afetada para longe do fator agressor até respostas emocionais complexas, expressas por padrões de comportamento inatos e aprendidos e sensações subjetivas de desconforto e sofrimento (Ferreira & Torres, 2004).

Avanços na pesquisa sobre plasticidade do sistema nervoso central têm tido marcadas repercussões no modo de abordar o fenômeno "dor". Um dos desenvolvimentos mais excitantes nessa área é o reconhecimento do papel de alterações na excitabilidade de neurônios centrais sobre a geração da hipersensibidade à dor (Woolf, 1996; Jacobson & Mariano, 2001). O conceito mais importante atualmente é o de que o SNC não é um sistema dividido em linhas de funcionamento rígido. É, sim, um sistema plástico, capaz de alterar estruturas e funções devido ao desenvolvimento orgânico normal e à influência da exposição a injúrias e a novas e velhas experiências (Woolf , 1996, McQuay & Dickenson, 1990).

As dores podem ser classificadas, segundo critério temporal, em agudas e crônicas. Dor aguda é causada por traumas, doenças subjacentes ou alterações funcionais musculares e viscerais. Está geralmente relacionada a processos inflamatórios (infecciosos ou não), espásticos e/ou isquêmicos. Tem importante objetivo fisiológico - alertar o indivíduo sobre algo que está errado, de modo que o organismo possa reagir a uma eventual agressão. Limitações funcionais determinadas pela dor previnem danos adicionais ou agravamento da patologia (Graeff, 1984, Morgan & Mikhail, 1996, Jacobson & Mariano, 2001). Caracteristicamente associa-se à resposta neuroendócrina ao estresse, que é proporcional à intensidade do estímulo. Tal resposta é mediada pelos sistemas nervoso simpático e endócrino. A ativação simpática aumenta o tônus aferente para

todas as vísceras e libera catecolaminas da medula adrenal. A resposta hormonal resulta do tônus simpático aumentado e de reflexos mediados pelo hipotálamo (Morgan & Mikhail, 1996; Gozzani, 1997).

Dor crônica é definida pela continuidade da percepção dolorosa por período prolongado, comumente por mais de 3 meses (Morgan & Mikhail, 1996, Jacobson & Mariano, 2001). Pode resultar de nocicepção periférica ou de disfunções de sistema nervoso central ou periférico (Dray *et al.*, 1994, Morgan & Mikhail, 1996, Wannmacher & Ferreira, 1998a). Vários eventos estão envolvidos na sua gênese - desde alterações de excitabilidade das fibras nervosas aferentes até marcadas mudanças de fenótipo celular, com expressão de novas moléculas, incluindo enzimas, neurotransmissores e receptores (Dray *et al.*, 1994, Jacobson & Mariano, 2001). Alterações centrais crônicas na neuroquímica da sinalização da dor levam à hipersensibilidade, que aumenta e prolonga níveis relativamente baixos de impulsos aferentes e permite que estímulos inócuos passem a ser percebidos como dor (Dray *et al.*, 1994, Morgan & Mikhail, 1996). Alterações estruturais, particularmente após lesões de nervos periféricos, também podem ser vistas, incluindo perda de interneurônios medulares, rearranjo inadequado de processos nervosos aferentes na medula espinhal e proliferação de fibras simpáticas em gânglios sensitivos (Dray *et al.*, 1994).

Quando há dano tecidual, ocorre liberação de substâncias endógenas, tais como serotonina, histamina, bradicinina e prostraglandinas que têm efeitos excitatórios sobre os nociceptores. Muitas dessas substâncias são simultaneamente neuroativas e vasoativas. E, por ação direta em membrana e/ou por alteração em seu micro-ambiente, ocorre excitação de nociceptores. Dependendo das substâncias predominantes, há prejuízo na microcirculação, pela presença de vasoconstrição ou

vasodilatação. Esses agentes promovem assim um ciclo vicioso de nocicepção - o aumento da permeabilidade vascular aumenta o extravasamento de substâncias algogênicas, mantendo as alterações no micro-ambiente dos nociceptores (Vane & Botting, 1995). A informação sobre o estímulo nocivo é enviada então ao sistema nervoso central por vias espinotalâmicas. Do tálamo partem vias que se dirigem ao córtex cerebral, permitindo a conscientização da dor (Ferreira & Torres, 2004). Paralelamente, o organismo é capaz de modular essa informação, em diferentes níveis, de modo que a dor percebida não é a "pior dor possível". Assim o indivíduo, além de ser capaz de reconhecer um estímulo nocivo, também é capaz de colocar em atividade um sistema de auto-analgesia (Jacobson & Mariano, 2001, Ferreira & Torres, 2004)

Modulação de vias de entrada (*inputs)* sensoriais (neste caso, relacionados à dor) ocorre em muitos níveis. Vias descendentes do hipotálamo, que possuem receptores sensíveis a opióides e são estimuladas por estresse emocional, podem transmitir sinais para o corno dorsal da medula espinhal, modulando a passagem de estímulos nociceptivos para centros superiores. Isso também pode ocorrer nos próprios centros superiores (por exemplo, em córtex frontal, mesencéfalo e bulbo) pela ação de opióides, antagonistas e agonistas de diversos neurotransmissores (Markenson, 1996).

A modulação periférica se expressa por meio do fenômeno de sensibilização. Após estimulação repetida, os nociceptores podem apresentar resposta aumentada a estímulos nocivos ou adquirir responsividade maior a quaisquer estímulos, incluindo os não-nocivos (Woolf, 1996). A chamada hiperalgesia primária caracteriza-se pela redução de limiar dos nociceptores, aumento da freqüência de resposta à mesma intensidade de estímulo, redução na latência dessa resposta e

ocorrência de disparos espontâneos, mesmo após a cessação dos estímulos (Woolf, 1996). É mediada pela liberação de substâncias algogênicas dos tecidos lesados, como histamina (liberada de mastócitos, basófilos e plaquetas) e serotonina (liberada de mastócitos e plaquetas) (Woolf, 1996, Wannmacher & Ferreira, 1998a). A histamina promove reação inflamatória local. Já a bradicinina ativa terminações nervosas livres por meio de receptores específicos (B_1 e B_2), levando ao efeito nociceptivo. A lesão tecidual leva à lise de células e à liberação de fosfolipídios de membranas celulares. Estes fosfolipídios, sob a ação da enzima fosfolipase A_2, formam ácido araquidônico. Por meio do sistema enzimático das cicloxigenases (COX-1 e COX-2), o ácido araquidônico é convertido em tromboxanas, prostaciclina e prostaglandinas. As prostaglandinas, especialmente PGE_2, sensibilizam os nociceptores periféricos às ações de histamina e bradicinina (Woolf, 1996; Wannmacher & Ferreira, 1998a). Pela via da lipoxigenase, ácido araquidônico é convertido em leucotrienos, que não têm papel bem definido na dor (Woolf, 1996).

A hiperalgesia secundária (ou inflamação neurogênica) também exerce importante papel na sensibilização periférica após lesões teciduais. Manifesta-se pela "tríplice resposta" - vermelhidão no sítio de lesão e ao seu redor (eritema), edema local e sensibilização ao estímulo nocivo. Deve-se à liberação de substância P em axônios colaterais de neurônios aferentes primários (daí a expressão "neurogênica"). A substância P é sintetizada e liberada por neurônios de primeira ordem, tanto na periferia quanto na medula espinhal, e facilita a transmissão da dor por meio da ação em receptores NK-1. Sensibiliza nociceptores e promove a liberação de histamina de mastócitos e serotonina de plaquetas. É potente vasodilatador, contribuindo para o edema tecidual. Induz a formação de leucotrienos

e atua atraindo leucócitos para o local da lesão (Woolf, 1996, Wannmacher & Ferreira 1998b).

Tem sido verificado que, após um período prolongado de estimulação, a hiperalgesia pode persistir, mesmo cessadas resposta inflamatória e dor. Neste caso, diante de um estímulo, mesmo leve, se restabelece o estado hiperalgésico anterior, fenômeno denominado de "memória periférica da dor" (Wannmacher & Ferreira 1998b).

No que se refere à modulação central, diversos mecanismos contribuem para o fenômeno de sensibilização em medula espinhal, sendo, portanto, facilitadores da dor. Estudos têm demonstrado que dano tecidual pode causar expansão de campos receptivos e diminuição no limiar de excitabilidade de neurônios do corno dorsal da medula, fazendo com que pequenos estímulos passem a desencadear respostas exageradas (alodinia), estímulos subliminares desencadeiem dor (hiperalgesia) ou haja até mesmo dor espontânea (Wannmacher & Ferreira, 1998b). Tal mecanismo contribuiria para o estabelecimento de dores crônicas, como a do membro fantasma, em que, mesmo após amputação, o paciente se queixa de dor com localização e intensidade similares às apresentadas antes da cirurgia (Wannmacher & Ferreira, 1998b).

No que se chama de expansão dos campos receptivos, os neurônios do corno dorsal aumentam seus campos de recepção de informações, de modo que as células adjacentes começam a se tornar também responsivas a estímulos para as quais antes eram irresponsivas (Woolf , 1996).

Os mecanismos responsáveis pela hiperexcitabilidade dos neurônios medulares têm sido um dos grandes alvos da atenção dos pesquisadores em Neurociências. Mendell e Wall, em 1965, relataram que, quando um nervo periférico

era estimulado com intensidade suficiente para ativar fibras C, a repetição de um estímulo fixo, em baixas freqüências, resultava em aumento progressivo da resposta (disparo do potencial de ação) em neurônios do corno dorsal da medula. Assim, mesmo sendo aplicado igual estímulo, a amplitude da resposta era cada vez maior. A esse fenômeno - incremento progressivo da resposta neuronal à estimulação repetida, deu-se posteriormente o nome de *wind-up* (que significa induzir um estado de grande tensão ou excitação). É um estado de hiperexcitabilidade dos neurônios de segunda ordem que se manifesta pelo aumento da freqüência de disparos com a repetição do mesmo estímulo e prolongamento desses disparos, mesmo após a estimulação das fibras aferentes C ter terminado. Poderia corresponder a uma "memória da dor", de forma similar ao fenômeno de potenciação de longa duração (*long term potentiation* ou LTP em inglês), que ocorre em estruturas cerebrais como hipocampo (Abraham & Williams, 2003). Assim como na LTP, os receptores glutamatérgicos exercem papel importante no estabelecimento da hiperexcitabilidade neuronal medular, e a administração de antagonistas específicos desses receptores abole o seu aparecimento (Willis, 2002).

Os mediadores neuroquímicos de sensibilização central incluem substância P, polipeptídeo intestinal vasoativo (VIP - *vasoactive intestinal polypeptide*), colecistocinina, angiotensina, galanina, glutamato e aspartato. Os dois últimos são aminoácidos excitatórios, sendo que o glutamato é considerado o principal neurotransmissor excitatório do sistema nervoso central (SNC). Glutamato e aspartato exercem importante papel no mecanismo de *wind-up*, por meio da ativação de receptores glutamatérgicos NMDA e não-NMDA. São considerados os principais responsáveis por indução e manutenção da sensibilização central. Quando estimuladas, as fibras C liberam substância P e glutamato, que co-existem nos

mesmos neurônios. Uma vez liberado, o glutamato atua sobre receptores glutamatérgicos NMDA, AMPA, cainato (KA) e metabotrópicos, levando ao aumento da concentração intracelular de cálcio em neurônios medulares e à ativação de fosfolipases C (PLC) e A_2 (PLA_2). A primeira catalisa a hidrólise de fosfatidil-inositol 4,5-bifosfato (PIP_2), gerando inositol trifosfato (IP_3) e diacilglicerol (DAG). O diacilglicerol atua como segundo mensageiro e ativa a proteína quinase C (PKC). A fosfolipase A_2 catalisa a conversão de fosfatidilcolina (PC) em ácido araquidônico (AA) e induz a formação de prostaglandinas. A ativação de receptores NMDA também induz a enzima óxido nítrico sintase, levando à formação de óxido nítrico. Tanto este quanto as prostaglandinas facilitam a liberação de aminoácidos excitatórios na medula espinhal, contribuindo para a manutenção da sensação dolorosa, mesmo quando os impulsos nociceptivos estão declinando na periferia (Woolf, 1996, Gozzani, 1997, Pleuvry & Lauretti, 1996, Dickenson, 1996, Wannmacher & Ferreira, 1998a e 1998b).

3. Estresse e nocicepção

Tem sido relatado que CRH induz a liberação de ACTH e β-endorfina da hipófise anterior (Vale *et al.*, 1981). β-endorfina e outros opióides endógenos estão envolvidos em uma série de funções e patologias, tais como dor, epilepsia, imunomodulação e estresse (Millan, 1986). β-endorfina hipotalâmica é liberada em resposta à novidade (Izquierdo, 1984; Izquierdo *et al.*, 1985), enquanto β-endorfina hipofisária, bem como encefalinas com origem em adrenal, são liberadas na circulação em resposta ao estresse (Lewis *et al.*, 1982, Hayden-Hixson & Nemeroff, 1993).

Analgesia induzida pelo estresse é fenômeno bem conhecido, e a função da β-endorfina no estresse tem sido bastante estudada (Amir *et al.*, 1980). Muitos estudos indicam que GCs e ACTH têm função não só no estresse como também em modulação da dor (Lewis *et al.*, 1980, MacLennan *et al.*, 1982) e excitabilidade cerebral (McEwen *et al.*, 1986). Uma série de investigações tem indicado que GCs, tais como a dexametasona, exercem consistente inibição sobre efeitos induzidos pela morfina em sensibilidade à dor e excitabilidade hipocampal. Há evidências de que ACTH e GCs reduzem a analgesia opióide (Chatterjee *et al.*, 1982). Dados indicam que, em cérebro de roedores, há importante interação funcional entre GCs e sistemas opióides, pelo menos em receptores μ. Já receptores δ e κ seriam modulados por outras vias (Pieretti *et al.*, 1994). Pré-tratamento com dexametasona foi capaz de reduzir analgesia induzida pela morfina, tanto em placa quente (Pieretti *et al.*, 1991) quanto no teste de *tail-flick* (Capasso *et al.*, 1992).

Exposição aguda a estressores produz diminuição na resposta a estímulos dolorosos (analgesia) (Amir & Amit, 1978, Menendez *et al.*, 1993). O organismo possui sistemas inibitórios da dor (auto-analgesia), em que opióides endógenos exercem papel importante (Vaccarino & Kastin, 2001). O estresse como indutor de analgesia (SIA) tem recebido grande atenção, por ser uma indicação do envolvimento de sistemas opióides endógenos em fenômenos comportamentais e adaptativos. Estudos têm sugerido que algumas formas de SIA são mediadas pelo sistema opióide, de modo que os animais desenvolveram tolerância cruzada para os efeitos analgésicos da morfina e os do estresse (Cancela *et al.*, 1995). Além disso, a analgesia pôde ser revertida por antagonistas opióides (Maier *et al.*,1980). No entanto, outras formas de analgesia induzida pelo estresse são mediadas por mecanismos não-opióides (Lewis,1986, MacLennan *et al.*,1982).

A descoberta de que a hipofisectomia reduz analgesia induzida pelo estresse mediada por opióide sugere que a β-endorfina pode mediar este tipo de analgesia (Amir *et al.*,1980). Além disso, há relatos de antinocicepção induzida pela novidade (o que leva à liberação desse opióide) (Netto *et al.*,1987, Dalmaz *et al.*,1991, Torres *et al.*, 2001b) e de que esta é revertida pelo antagonista opióide naltrexona (Siegfried *et al.*, 1987).

Resultados obtidos em nosso laboratório mostraram que ratos controles apresentam antinocicepção induzida pela novidade, o que concorda com dados da literatura, sugerindo que certas formas de novidade podem alterar a sensibilidade à dor (Fanselow, 1985; Netto *et al.*, 1987). Por outro lado, quando animais cronicamente estressados foram testados, não houve diferença significativa na resposta nociceptiva, comparando-se as situações de ausência e presença de novidade (Torres *et al.*, 2001b). Visto que a antinocicepção induzida pela novidade em ratos, avaliada por meio do aparelho de *tail-flick*, tem sido sugerida como mediada pela liberação de β-endorfina (Siegfried *et al.*, 1987; Netto *et al.*, 1987), a ausência deste efeito sugere alteração no padrão de resposta do sistema opióide nos animais imobilizados cronicamente.

Dados prévios do laboratório também indicam que ratos machos estressados cronicamente por imobilização apresentam diminuição na latência de retirada da cauda em resposta a um estímulo térmico nocivo (*tail-flick*), caracterizando uma resposta hiperalgésica, tanto na medida basal quanto após exposição a uma nova sessão de imobilização (Gamaro *et al.*, 1998, Torres *et al.*, 2001b). Quando ratas foram testadas, não responderam ao estresse crônico com hiperalgesia (Gamaro *et al.*, 1998), sugerindo que este resultado é dependente do sexo do animal.

A hiperalgesia observada em machos mostrou-se reprodutível, visto que animais cronicamente estressados utilizados em experimentos posteriores, mesmo com diferentes modelos de estresse crônico (Gamaro, 1998, Torres *et al.*, 2001b), também apresentaram hiperalgesia.

Investigou-se posteriormente o efeito da suspensão do estresse por imobilização sobre a nocicepção (Torres, 1999). Tanto 14 quanto 28 dias após a interrupção do tratamento, os animais continuavam hiperalgésicos em relação aos controles. Submetidos a um novo estímulo estressante (3 h de imobilização), observou-se presença de antinocicepção nos animais controle, conforme era esperado, enquanto que, somente após 28 dias após a suspensão, o efeito antinociceptivo dessa nova exposição ao estresse foi observado nos animais estressados. É possível que a ausência de resposta a uma nova exposição ao estresse, observada nos animais cronicamente estressados com 14 dias de suspensão do tratamento, deva-se a uma adaptação ao agente estressor. É sabido que a exposição repetida ao estresse pode causar adaptação e diversos neurotransmissores têm sido implicados nesse fenômeno. Por outro lado, esse efeito seria de natureza diferente daquele envolvido na gênese da resposta hiperalgésica, uma vez que o seu desaparecimento não segue o mesmo curso temporal - a hiperalgesia permaneceu após 28 dias de suspensão do estresse, mas a resposta a um estressor agudo voltou mais precocemente a ser similar à do grupo controle. Assim, é possível que mais de um sistema esteja envolvido nas adaptações ao estresse crônico no que tange a seus efeitos nociceptivos.

Potenciação de magnitude e duração do efeito analgésico de fármacos opióides, em ratos expostos a estresse agudo por imobilização, têm sido demonstradas. É possível que o mecanismo responsável por este efeito envolva

sítios espinhais e supraespinhais (Calcagnetti & Holtzman, 1992). Essa conclusão é suportada pela observação de que o efeito de agonistas opióides, administrados por via intracerebroventricular, é aumentado em ratos estressados por imobilização (Calcagnetti *et al.*, 1992). Torres (1999) observou que ratos cronicamente estressados não apresentaram o efeito analgésico característico após injeção de morfina (1 e 5 mg/kg, por via i.p.), sugerindo que o estresse crônico pode provocar alterações em sistemas opióides endógenos, o que concorda com estudos prévios (para revisão ver Drolet *et al.*, 2001). Mudança na sensibilidade a opióide observada nesses ratos pode ser decorrente de alterações em receptores opióides centrais ou periféricos, tanto em número quanto em afinidade. Por outro lado, essa mudança pode ser determinada por alterações em outros sistemas hormonais ou de neurotransmissores capazes de interagir com receptores opióides. Em modelo de estresse por imobilização, observou-se diminuição na densidade de receptores opióides em sistema nervoso central (Dantas, 2002). Uma vez que a morfina exerce seus efeitos nociceptivos primariamente por meio de receptor opióide μ, a alteração observada em animais submetidos a estresse repetido por imobilização pode ser atribuída a mudanças nos níveis desses receptores.

4. Sistema purinérgico

4.1. Adenosina trifosfato - ATP

Além de seu papel central em metabolismo celular energético, ATP é um neurotransmissor, encontrado em grânulos secretórios de neurônios e células adrenais cromafínicas, liberado em "quanta" em resposta a potenciais de ação (Linden, 1999).

O ATP é liberado de sinaptossomas de córtex, hipotálamo e bulbo. Em sinaptossomas corticais, é co-liberado com acetilcolina e noradrenalina, mas, em geral, é liberado de neurônios não-colinérgicos e não-adrenérgicos (Linden, 1999, Burnstock, 1999). É também co-transmissor de neurônios gabaérgicos (Burnstock, 1999). É liberado em fluido extracelular como resultado de lise celular, permeabilização seletiva de membrana ou exocitose de grânulos secretórios, podendo exercer funções completamente diferentes da função de mediador intracelular.

ATP é neurotransmissor em sistemas simpático, parassimpático e nervos sensoriais periféricos, assim como em SNC (Cunha & Ribeiro, 2000). Além de liberação neuronal como transmissor ou co-transmissor, há outras fontes não neuronais de liberação de ATP incluindo plaquetas, células mononucleadas, células endoteliais, e lise celular (Linden, 1999).

A inativação metabólica extracelular do ATP produz outras purinas, incluindo ADP, AMP e adenosina (ADO), formando a cascata purinérgica (figura 2).

ATP extracelular e os produtos resultantes de sua quebra (ADP e adenosina) têm pronunciados efeitos em uma variedade de processos biológicos, incluindo neurotransmissão, contração muscular, funções cardíaca e plaquetária, vasodilatação e metabolismo hepático do glicogênio (Agteresch *et al.*,1999).

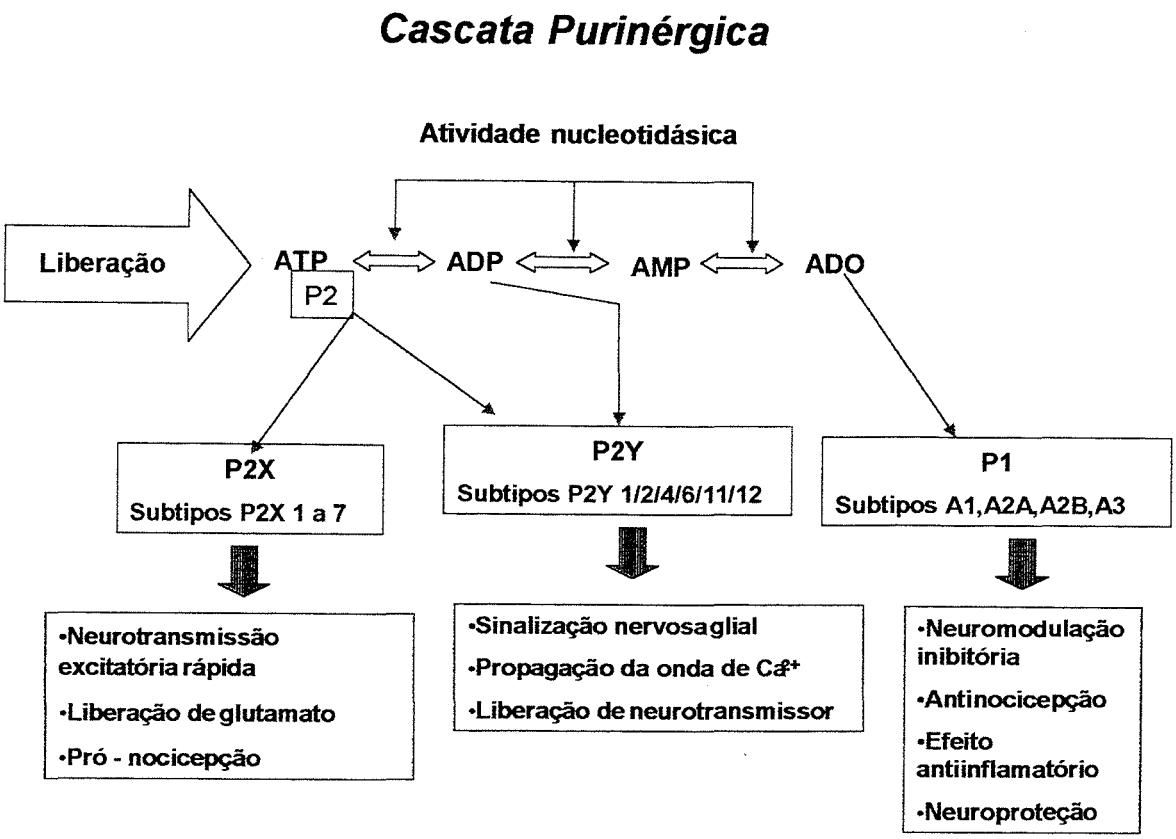

Figura 2 –Cascata purinérgica. (Figura adaptada de Jarwis & Kowaluk, 2001).

Dessa forma, ATP, ADP e adenosina (ADO) são liberados de células no espaço extracelular e interagem com famílias de receptores específicos de superfície celular. Os receptores P1, em que se liga adenosina, apresentam dois subtipos – A1 e A2. Os denominados P2, em que se ligam ATP e ADP, subdividem-se em 5 subtipos P2X, P2Y, P2T, P2U, P2Z (tabela 4). Esses receptores têm sido descritos em vários tecidos, como os tecidos nervoso, pulmonar, ósseo, cartilaginoso e sangüíneo (Burnstock & Williams, 2000). Sua ativação leva a diferentes repercussões sobre a neurotransmissão sensorial. No caso da ADO, esta funciona como freio homeostático inibitório às ações excitatórias do ATP.

A ativação de receptor P1 de adenosina diminui nocicepção, inflamação e excitabilidade celular, enquanto a ativação de receptor P2X por ATP estimula a excitabilidade celular, aumenta a liberação de aminoácidos excitatórios, inicia respostas nociceptivas e pode levar à apoptose (Burnstock & Williams, 2000). Já ativação de P2Y por ATP/ADP pode facilitar a neurotransmissão excitatória pela modulação da atividade neuronal dependente de cálcio (Fam *et al.*, 2000).

Tabela 4: Receptores purinérgicos (Linden, 1999).

Nucleotídeo	**Receptor**	**Efetor**
Adenosina	A1	Inibe adenilato ciclase
		Aumenta a condutância de canal de K^+
		Diminui a condutância de canal de Ca^{2+}
		Aumenta ou diminui a atividade de fosfolipase C
	$A2_a$	Ativa adenilato ciclase
	$A2_b$	Ativa fosfolipase C
ATP	P2X	Aumenta a condutância de canal de Ca^{2+}
	P2Z ($P2X_7$)	Aumenta a condutância de canal iônico
ATP/ADP	P2Y	Ativa fosfolipase C
		Ativa fosfolipase D
	P2U ($P2Y_2$)	Ativa fosfolipase C
	P2T ($P2Y_T$/$P2Y_{12}$)	Inibe adenilato ciclase

O ATP administrado sistemicamente produz respostas de dor, e o ATP endógeno pode contribuir para dor associada com causalgia, distrofia simpática

reflexa, angina, enxaqueca, dor lombar, pélvica e de câncer (Burnstock, 2000). Papel específico para o ATP como neurotransmissor em sinalização de dor é baseado, em parte, no fato de ser liberado de nervos sensoriais e em dados mostrando que o ATP produz potenciais excitatórios rápidos em neurônios do gânglio dorsal (Jarvis & Kowaluk, 2001).

Na periferia, o ATP pode ser liberado de fontes neuronais ou não-neuronais. Fontes neuronais incluem neurônios aferentes sensoriais, simpáticos e parassimpáticos. Após a liberação, ATP tem potencial para modificar respostas pela ativação de receptores P2 (pré- e pós-sinápticos) ou, após sua quebra, de receptores P1 por ADO. O ATP pode também ser liberado de células musculares esqueléticas e lisas, vasculares (hemácias, células mononucleares e plaquetas), endoteliais, epiteliais e certos tipos de células secretórias. Os mecanismos de liberação incluem exocitose de ATP granular ou vesicular e liberação citosólica, o que pode ocorrer durante lesão ou inflamação tecidual (para revisão ver Sawynok & Liu, 2003).

Considerando aspectos fisiopatológicos, as ações de ATP e ADP em paredes vasculares, em particular no endotélio, são especialmente relevantes, uma vez que grandes quantidades desses nucleotídeos estão estocadas em plaquetas e liberadas durante a agregação plaquetária (Motte *et al.*,1995). Em neurônios de sistema nervoso central e periférico, o ATP é co-localizado e co-secretado com muitos neurotransmissores, como noradrenalina, acetilcolina, glutamato, ácido γ-aminobutírico (GABA) (Burnstock, 1999).

Nucleotídeos extracelulares, tais como ATP, ADP e ADO, são sabidamente reguladores da resposta vascular à injúria endotelial. O ATP liberado como co-transmissor de terminais nervosos simpáticos atua em purinoceptores P2X para

produzir vasoconstrição. O ATP é também liberado por cisalhamento *(sheer stress)* e hipóxia de células endoteliais, atuando em receptores P2Y nessas células e liberando óxido nítrico (NO), com subseqüente vasodilatação. O ADP é um potente fator recrutador de plaquetas e indutor de agregação plaquetária por meio de interação com dois subtipos de receptores P2 plaquetários - receptores $P2Y_1$, ligados à via da fosfolipase C e a influxo de cálcio e envolvidos com alteração da forma plaquetária e sua agregação transitória; receptores P2T ($P2Y_T/P2Y_{12}$), negativamente ligados à adenilato ciclase que medeiam degranulação e agregação sustentada (Burnstock & Willians, 2000). A hidrólise do ADP inibe a agregação plaquetária por remoção do ADP e formação de ADO (Zimmermann, 1996).

O ATP é antagonista competitivo do ADP em receptores P2Y de plaquetas e estimula a produção de PGI_2 e NO, que inibem a agregação plaquetária e atuam como vasodilatadores.

Trabalhos têm demonstrado *in vitro* que altas concentrações de ATP inibem a agregação plaquetária induzida pelo ADP (Leon *et al.*, 1997, Park *et al.*, 1999). No entanto, baixas concentrações (0,01-1,0 μmol/L) podem aumentar agregação plaquetária induzida por colágeno e trombina, (Soslau *et al.*, 2000). Além disso, ativação de receptores de canais de cálcio ligados a ATP ($P2X_1$) nas plaquetas estimulam a mudança na forma da plaqueta, sugerindo que ATP contribua para ativação plaquetária (Rolf *et al.*, 2001).

Múltiplos receptores e alças de retroalimentação negativa estão envolvidos em regulação local do tônus vascular e homeostasia pelos nucleotídeos da adenina (figura 3). Grandes quantidades de ATP e ADP são liberadas localmente de plaquetas em resposta a agentes agregantes. ADP recruta plaquetas adicionalmente, por meio de ativação de receptores P2T. Por outro lado, ATP e ADP

liberados de plaquetas ou células endoteliais ativam receptor P2 (P2Y e P2u) expressos em células endoteliais. Isso resulta em liberação de dois potentes vasodilatadores e inibidores de agregação plaquetária – prostaciclinas (PGI_2), que atuam por meio de receptor especifico (IP), e óxido nítrico (NO). O ATP atua em receptores P2Y de células endoteliais, liberando óxido nítrico, com subseqüente vasodilatação. O músculo liso pode ser diretamente contraído por ATP que atua em receptores P2X. Finalmente, o ATP é rapidamente degradado por ectonucleotidases endoteliais em adenosina a qual inibe a agregação plaquetária e induz relaxamento de músculo liso por meio de receptores A2 (Motte *et al.*, 1995). A CD39 (uma ATP-difosfo-hidrolase; vide abaixo) atua junto a nucleotídeos extracelulares liberados durante dano tecidual, modulando fluidez sangüínea e ativação plaquetária (Zimmerman, 1996). Esses achados sugerem uma complexa função para o ATP na regulação da agregação plaquetária e que a ação do ATP é dependente da sua hidrólise na circulação.

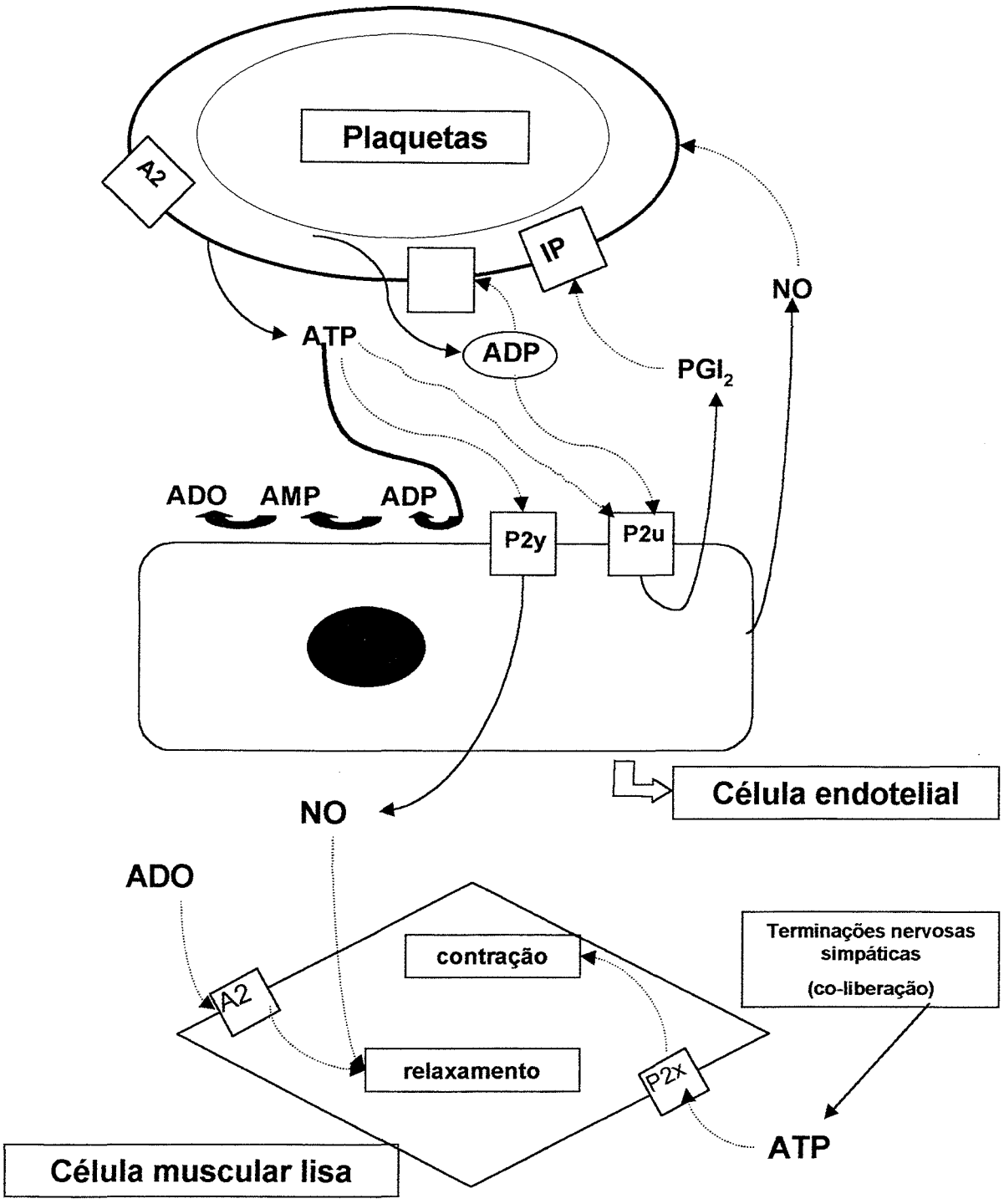

Figura 3: Regulação local de tônus vascular e hemostasia por nucleotídeos de adenina (Motte *et al.*, 1995).

4.2. Ectonucleotidases

ATP, assim como outros agonistas de nucleotídeos para P2-puriceptores, podem ser degradados por ectonucleotidases (Zimmermann, 1996).

Ectonucleotidases estão localizadas na superfície celular; nucleotidase também podem apresentar-se solúveis em meio intersticial ou fluidos biológicos(Zimmermann, 2001). Nucleotidases solúveis, que hidrolisam ATP a ADO, também são liberadas de nervos simpáticos (Todorov *et al.*,1997). Trabalhos prévios demonstraram que membros de várias famílias de ectonucleotidases podem contribuir para a hidrólise de nucleotídeos. Nucleosídeos 5'tri- e difosfatos (NTP e NDP) podem ser hidrolisados por membros das famílias de E-NTPDase (ectonucleosídeo trifosfato difosfo-hidrolase), E-NPP (ectonucleosídeo pirofosfatase/fosfodiesterase) e fosfatases alcalinas (Zimmermann, 2001). Estas ectonucleotidases, junto com 5´-nucleotidase, controlam a disponibilidade de ligantes (ATP, ADP, AMP e ADO) para receptores de nucleotídeo e nucleosídeo e, conseqüentemente, duração e extensão da ativação do receptor (Chen & Guidotti, 2001). Portanto, a cascata de ectonucleotidases é via enzimática com dupla função - remover um sinal (ATP) e gerar um segundo (ADO). Pode ter função na regulação efetiva de vários processos, uma vez que tem considerável plasticidade em diferentes situações patogênicas (Agteresch *et al.*,1999). Além disso, regula uma variedade de estados fisiológicos, incluindo função cardíaca, secreção hormonal, resposta imune, neurotransmissão e agregação plaquetária, por meio de modulação dos níveis circulantes de nucleotídeos no sangue (Todorov *et al.*, 1997, Marcus *et al.*, 1997, Agteresch *et al.*,1999, Mulero *et al.*,1999). Podem também ter função de proteção pela manutenção dos níveis extracelulares de ATP/ADP e ADO dentro de patamares fisiológicos (Agteresch *et al.*, 1999).

As ectonucleotidases hidrolisam uma variedade de nucleosídeos da purina e pirimidina di- e tri-fosfatos. São ativadas por altas concentrações de Ca^{2+} e Mg^{2+}. Atuam em pH ótimo alcalino e não são inibidas por inibidores típicos de ATPases intracelulares, tais como as ATPases dos tipos P, F e V ou fosfatases alcalinas (Zimmermann, 1996). Ecto-ATPase tem maior afinidade pelo ATP, enquanto a ecto-ATP-difosfo-hidrolase (ecto-apirase) hidrolisa igualmente ATP e ADP.

Análises moleculares revelaram que as duas ectonucleotidases (ecto-ATPase e ecto-ATPdifosfo-hidrolase – tabela 5) têm larga distribuição tecidual. Ambas são expressas em cérebro e uma variedade de tecidos, incluindo coração, rins, baço, pulmões e músculo esquelético (Zimmermann *et al.*,1998). A ecto-ATPdifosfo-hidrolasenpode inibir a formação de trombos por meio da hidrólise do ADP.

Alguns estudos têm sugerido que nucleotidases solúveis são liberadas juntamente com ATP de nervos simpáticos (Todorov *et al.*, 1997). Há crescente interesse na possibilidade de que inativação extra-celular do ATP e formação de ADO aumentem a atividade nervosa por meio de receptores A2. A fonte celular da liberação de ecto-nucleotidases e a identidade molecular das enzimas não são conhecidas. A forma da ecto-5'-nucleotidase capaz de desprender-se da membrana está ancorada a glicosilfosfatidil inositol (GPI) (Zimmermann, 1992). Um processo proteolítico específico deve ser requerido para a liberação dessa enzima. Por outro lado, a ecto-ATPase e a ecto-ATPdifosfo-hidrolase parecem estar ancoradas à membrana por dois domínios transmembranas.

A ecto-5`-nucleotidase ocorre em todos os tecidos e hidrolisa os nucleosídeos 5`-monofosfato (NMP), tais como AMP. A função maior dessa enzima é a produção extracelular de nucleosídeo.

Tabela 5: Nomenclatura da família de E-NTPDase (Zimmermann, 2001)

Enzima	Outras denominações	Localização	Hidrólise
NTPDase 1	CD39, ecto-ATP difosfo-hidrolase, ecto-apirase	Superfície celular	Hidrolisa igualmente ATP e ADP
NTPDase 2	CD39L1, ecto-ATPase	Superfície celular	Hidrolisa ATP e ADP com preferência 30 vezes maior pelo primeiro
NTPDase 3	CD39L3, HB6	Superfície celular	Hidrolisa ATP e ADP com preferência 3 vezes maior pelo primeiro
NTPDase 4	UDPase (hLALP70v, LALP70)	Complexo de Golgi, Vacúolo lisossomal/ Autofágico	Hidrolisa pouco ATP e ADP
NTPDase 5	CD39L4, ER-UDPase	Retículo endoplasmático, Solúvel meio intersticial ou fluidos biológicos	Alta preferência por Nucleosídeos difosfatados
NTPDase 6	CD39L2	Complexo de Golgi, Solúvel meio intersticial ou fluidos biológicos	Alta preferência por Nucleosídeos difosfatados

4.4 ADENOSINA

Além do ATP, ADO (produto da hidrólise do ATP) também tem várias funções no SNC. Exerce tônus inibitório sobre neurotransmissão e ações neuroprotetoras em condições patológicas (Latini & Pedata, 2001). É particularmente usada como mensageiro transcelular para sinalizar desequilíbrio metabólico. Vários relatos têm documentado aumento na sua concentração em alterações metabólicas estressantes (Latini & Pedata, 2001), e o termo "*retaliatory metabolite*" tem sido utilizado para caracterizar a função homeostática da ADO que ocorre em virtualmente todos os tipos celulares (para uma revisão sobre adenosina, ver Cunha, 2001).

A Adenosina é potente neuromodulador endógeno que parece atuar como agente neuroprotetor, regulador da suscetibilidade a convulsões e analgésico endógeno (Cunha, 2001). Participa da regulação do controle sensório-motor (Ferré *et al.*, 1992) e é modulador dos processos de dor na medula espinhal (Sawynok & Sweeney,1989). Seus receptores estão concentrados em substância gelatinosa do corno dorsal da medula espinhal (Geiger, *et al.*, 1984). Receptores A1 de ADO são encontrados em neurônios e estão envolvidos em regulação e liberação de aminoácidos excitatórios. ADO pode exercer ação inibitória na transmissão sináptica nociceptiva em medula espinhal e cérebro (Sawynok & Sweeney, 1989, Sollevi, 1997).

Administração de inibidores de ADO quinase e agonista s A1 produz potentes efeitos em SNC, podendo ser alternativa terapêutica no tratamento de doenças neurodegenerativas mas exerce diminuída influência no sistema cardiovascular, (Zalewka-Kaszubska, 2002). Recentemente têm sido relatados efeitos deletérios após a ativação de receptores de ADO subtipos A2a e A3. Por outro lado, a

administração de antagonista seletivo de receptor A2A determinou marcada redução na morte celular após isquemia cerebral em ratos (Zalewka-Kaszubska, 2002). ADO é produzida em cardiomiócitos e células endoteliais e é cardioprotetora. A ativação de receptores A1 resulta em atenuação da liberação de catecolaminas, minimizando o aumento da contração miocárdica mediada por adrenoceptores. Por sua vez, a ativação de receptores A2 determina aumento de fluxo sangüíneo, inibição de plaquetas e de ativação de leucócitos (Kitakaze et al., 1999).

ADO em condições fisiológicas exerce ação no cérebro, principalmente por meio de receptores A1 e A2 (Cunha, 2001). Receptores A1 são largamente distribuídos no SNC, com altos níveis de expressão em áreas ricas em receptores para GCs, como hipocampo, córtex cerebral e córtex cerebelar (Goodman & Snyder, 1982, Fastbom *et al.*,1987). Há dados da literatura que sugerem que GCs podem regular receptores de ADO no cérebro. Há também algumas evidências de que estresse agudo, que sabidamente pode afetar níveis de GCs, pode influenciar receptores A1 no SNC (Boulenger *et al*, 1984). Estudos de Svenningsson e Fredholm (1997) demonstraram que GCs endógenos e exógenos regulam o número de receptores A1, pelo menos em parte, pela alteração da expressão de RNAm em cérebro de rato. Ao contrário, nenhum efeito foi encontrado em receptores A2 ou em seu RNAm. Pode-se especular que as alterações no receptor A1 determinadas por GCS têm conseqüências funcionais, pois esses receptores são sabidamente moduladores de muitas funções cerebrais.

Como já mencionado, a concentração de ADO extracelular têm se mostrado aumentada em condições estressantes (Latini & Pedata, 2001), incluindo exposição a choque (Minor *et al.*, 2001). A hidrólise de nucleotídeos tem sido relacionada com

grande número de processos fisiológicos, e vários deles podem ser alterados por estresse.

OBJETIVOS

Tendo em vista o que foi exposto previamente, os objetivos dessa tese são:

- avaliar o efeito de estresse crônico por imobilização sobre a resposta nociceptiva resultante da exposição a diferentes estressores;
- avaliar o papel modulatório de sistema purinérgico, por meio da administração de agonista e antagonista específicos, sobre a resposta nociceptiva de ratos submetidos a estresse crônico por imobilização;
- avaliar o efeito de estresses crônico e agudo por imobilização sobre as atividades ATPásica e ADPásica e 5'nucleotidase em sinaptossomas de medula espinhal, em ratos machos e fêmeas;
- avaliar o efeito de estresse crônico por imobilização sobre as atividades ATPásica e ADPásica e 5'nucleotidase em sinaptossomas de hipotálamo e córtex cerebral e em soro de ratos;
- avaliar o efeito do estresse agudo sobre as atividades ATPásica e ADPásica e 5'nucleotidase em soro de ratos, em diferentes tempos após exposição a estresse por imobilização.

ORGANIZAÇÃO DOS TRABALHOS QUE COMPÕEM ESSA TESE

Capítulo I - Long-lasting delayed hyperalgesia after chronic restraint stress in rats - effect of morphine administration. *Neuroscience Research* 2003; 45: 277-283.

Nesse estudo, foram avaliados os efeitos da administração de morfina em animais estressados cronicamente por imobilização e aqueles decorrentes da interrupção do estresse crônico por 14 e 28 dias, com posterior exposição a estresse agudo de imobilização. Foi comparada a resposta nociceptiva de animais controles e estressados no aparelho de *tail-flick*. Nessa tese, foram acrescentados os resultados obtidos com a exposição desses animais a diferentes tipos de estresse agudo sobre a nocicepção.

Capítulo II - Effect of drugs active at adenosine receptors upon chronic stress-induced hyperalgia in rats. *European Journal of Pharmacology* 2003; 481: 197-201.

Nesse estudo, investigou-se o efeito da administração de agonista de receptor A1 de adenosina, N^6-ciclopentildelxantina (CPA), e de antagonista desse mesmo receptor, 1,3-dipropil-8-ciclopentilxantina (DPCPX), bem como o efeito do bloqueio da captação de adenosina com dipiridamol, sobre hiperalgesia induzida por estresse crônico em ratos. Foi avaliado o papel da adenosina nesse processo.

Capítulo III – Effect of chronic and acute stress on ectonucleotidase activities in spinal cord . *Physiology & Behavior* 2002; 74: 1-5.

Nesse trabalho analisaram-se os efeitos de estresses agudo e crônico sobre as atividades ATPásica-ADPásica e de 5`nucleotidase em sinaptossomas de medula espinhal de ratos machos e fêmeas.

Capítulo IV – Chronic stress effects on adenine nucleotide hydrolysis in the blood serum and brain structures of rats. *Pharmacology Biochemistry and Behavior* 2002; 74: 181-186.

Nesse estudo, investigou-se o efeito do estresse crônico por imobilização sobre a hidrólise dos nucleotídeos da adenina (atividades ATPásica, ADPásica e de 5`nucleotidase) em sinaptossomas de duas estruturas cerebrais (córtex frontal e hipotálamo) e em soro de ratos machos.

Capítulo V – The effect of stress upon hydrolysis of adenine nucleotides in blood serum of rats. *Pharmacology Biochemistry and Behavior* 2003; 75: 467-471.

Nesse estudo, investigou-se o efeito do estresse agudo por imobilização sobre a hidrólise dos nucleotídeos da adenina (atividades ATPásica-ADPásica e de 5`nucleotidase) em soro de ratos machos sacrificados 0, 6, 24 e 48 horas após a sessão de estresse.

CAPÍTULO I

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Long-lasting delayed hyperalgesia after chronic restraint stress in rats—effect of morphine administration

Iraci Lucena da Silva Torres [a,*], Simone N.S. Cucco [a], Marcio Bassani [a], Marcelo S. Duarte [a], Patricia P. Silveira [a], Ana Paula Vasconcellos [a], Angela Sampaio Tabajara [a], Giovana Dantas [a], Fernanda U. Fontella [a], Carla Dalmaz [a], Maria Beatriz C. Ferreira [b]

[a] *Departamentos de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600-Anexo, Lab. 32, 90035-003 Porto Alegre, RS, Brazil*
[b] *e Farmacologia Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

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Abstract

Different effects upon the nociceptive response have been observed with exposure to acute and chronic stress in rats. In the present study we repeatedly submitted rats to restraint for 40 days, inducing hyperalgesia using the tail-flick test. A new session of acute stress was applied at the end of 40 days period, and the chronically-stressed animals demonstrated analgesia after forced swimming, but not after restraint. The effect of stress interruption for 14 or 28 days on the nociceptive threshold was then investigated. The basal tail-flick latency remained decreased for at least 28 days (hyperalgesic effect). Following the periods of suspension, the animals were submitted to new session of acute restraint, and stress-induced analgesia was observed only after 28 days of stress interruption. Thus, the mechanisms involved in the long-lasting hyperalgesia presented in this study are not exactly the same as those responsible for the analgesia induced by acute stressors. After 40 days of chronic stress treatment, morphine was injected i.p. (1.0, 5.0 mg/kg or saline). The repeatedly stressed rats displayed decreased morphine effects on nociception compared to unstressed controls. The tolerance of the response to morphine agrees with previous studies suggesting that chronic restraint stress could modify the activity of opioid systems.

Keywords: Hyperalgesia; Nociception; Chronic stress; Restraint; Morphine; Tail-flick; Rats

1. Introduction

It is well known that stress produces a series of physiological and behavioral changes that enable the individual to cope with new situations, and several neurotransmitter and hormonal systems are known to be affected by stressful events. Conversely, animals submitted repeatedly to a stress situation do not experience all the hormonal consequences that animals exposed to one single stress episode experience (Hashiguchi et al., 1997; Torres et al., 2001a), and this phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes. Various neurotransmitter systems have been suggested to play a role in this stress desensitization process, including opioid modulation (Cancela et al., 1988, 1991, 1995).

Physiological responses to stress include alterations in the perception and response to pain. Acute exposure to a variety of stressors produces immediate analgesia in several pain tests (SIA) (Vacarino and Kastin, 2001). Some studies, though, have reported that under some experimental conditions both acute and chronic stress can elicit hyperalgesia instead of analgesia (Vidal and Jacob 1982; Satoh et al., 1992; Quintero et al., 2000). For example, brief exposure to short emotionally-arousing non-noxious stress, such as holding or novel

* Corresponding author. Tel.: +55-51-3316-5577; fax: +55-51-3316-5540.
E-mail address: torresf@zaz.com.br (I.L. da Silva Torres).

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environments, produces an immediate and transient hyperalgesia, followed by longer analgesia in response to thermal and electrical stimuli in rats (Vidal and Jacob, 1982). In addition, prolonged stress by repeated exposure to a cold environment or to restraint induces hyperalgesia (Satoh et al., 1992; Gamaro et al., 1998).

Previous data from our laboratory showed decreased pain thresholds after exposure to repeated restraint stress in male rats (Gamaro et al., 1998). In addition, whilst control rats presented novelty-induced antinociception, which has been attributed to opioid activation (Netto et al., 1987; Siegfried et al., 1987; Dalmaz et al., 1991), chronically-stressed groups showed no significant difference between pre- and post-novelty tail-flick latencies (TFL) (Torres et al., 2001b).

Opioid receptors have considerable plasticity as reflected by their susceptibility to modifications by various pharmacological and behavioral manipulations (for review, see Drolet et al., 2001). Chronic exposure to antagonists causes receptor up regulation, whilst chronic exposure to agonists down regulates receptor number (Belcheva et al., 1998). In animals submitted to acute stress, a potentiation of the magnitude and duration of the analgesic effect of some opiate agonists has been demonstrated (Calcagnetti and Holtzman, 1992). Although the precise mechanisms involved in the development of hyperalgesia observed after repeated restraint are not known, it is suggested that they could be related, at least in part, to alterations in the central or peripheral opioid activity (Gamaro et al., 1998; Torres et al., 2001b), and the absence of novelty-induced antinociception in these animals supports this theory. Therefore, one of the aims of the present work is to verify the effects of chronic restraint stress on morphine-induced antinociception, as measured by the tail-flick test. The effect of exposure to two different forms of acute stress in chronically-stressed animals and controls is also reported.

Although hyperalgesia induced by repeated stress has been reported (Satoh et al., 1992; Gamaro et al., 1998), it is not known how long this effect lasts after the suspension of the stress regimen. Another objective of this study is to verify the effect of the suspension of chronic restraint stress for 14 or 28 days upon the nociceptive threshold, as measured by the tail-flick test. The nociceptive response to the exposure to acute restraint stress in control and chronically-stressed rats was also analyzed 14 or 28 days after stress suspension.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (60 days at the beginning of the treatment, 200–230 g) from our breeding stock were used. Experimentally naive animals were housed in groups of 5 in home cages made of Plexiglas material ($65 \times 25 \times 15$ cm^3) with the floor covered with sawdust. They were maintained on a standard 12-h dark/light cycle (lights on between 7.00 and 19.00 h) at room temperature (22 ± 2 °C). The rats had free access to food (standard lab rat chow) and water, except during the period of exposure to the stressor. All animal procedures were approved by the institutional Research Committee, and measures were taken to minimize pain and discomfort.

2.2. Chronic restraint stress procedure

The animals were stressed by restraint 1 h daily, 5 days per week for 40 days (Ely et al. 1997). Restraint was carried out by placing the animal in 25×7 cm^2 plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole in the far end for breathing. The control group was not submitted to stress, being kept in their home cages. The immobilization procedure was performed between 10:00 and 12:00 h.

2.3. Tail-flick measure

Nociception was assessed with the tail-flick apparatus (D'Amour and Smith, 1941). Rats were wrapped in a towel and placed on the apparatus. The light source positioned below the tail was focused on a point 2–3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. The TFL represented the period of time (seconds) from the beginning of the trial to the tail deflection. A cut-off time of 10 s was used to prevent tissue damage. Shortly after the last session of the treatment (40 days) and 24 h before the first measurement, the animals were exposed to the tail-flick apparatus to familiarize them with the procedure, since the novelty of the apparatus can itself induce antinociception (Netto et al., 1987).

2.4. Acute stress models

The Wistar rats were repeatedly restrained during 40 days. After this period, both control and stressed groups were submitted to two forms of acute stress. TFL were obtained before and immediately after exposure to this new stress session. The stressors applied were 3 h restraint (Ely et al., 1997) ($n = 10$–15/group) or forced swimming ($n = 12$–15/group, carried out by placing the animal in a glass tank measuring $50 \times 47 \times 40$ cm^3, with 30 cm of water at 20 °C for 10 min) (Terman et al., 1986). Restraint session was omitted only in the days when another stressor was used.

2.5. Effect of suspension of stress for 14 or 28 days

Rats were submitted to chronic stress by repeated restraint or were maintained in their home cages as described above. The stress treatment was then interrupted for 14 ($n = 7$–8/group) or 28 days ($n = 15$–16/group). Different groups of animals were used to assess nociception after these periods of interruption. After both periods, the animals were submitted to one restraint session lasting 3 h. The nociceptive response (TFL) was evaluated at 3 times: (a) at the end of the chronic stress treatment; (b) at the end of the suspension period; and (c) after the acute exposure to the stressor.

2.6. Morphine administration

The Wistar rats were submitted to chronic stress as described above. After 40 days of treatment, the animals were familiarized with the tail-flick apparatus.

On the next day, morphine 1.0 mg/kg ($n = 8$–10/group), 5.0 mg/kg ($n = 8$/group) (D'Amato et al., 1999) or saline ($n = 9$–13/group) was injected i.p. TFL was measured before, 30 and 60 min after the injection. Morphine sulfate was dissolved in 0.9% saline and administered i.p. in a volume of 1.0 ml/kg.

3. Statistical analysis

Data were expressed as median (interquartile range) of TFL. The comparison between two groups was made by Wilcoxon Matched-Pairs Signed-Ranks test (for repeated measures) or by Mann–Whitney U-test (for independent samples). The comparison of more than two dependent groups (morphine effect analysis) was made by Friedmann-ANOVA test.

4. Results

4.1. Effect of different forms of acute stress upon the nociceptive response in chronically-stressed rats

Results are shown in Fig. 1. According to the basal measures, the chronically-stressed animals were hyperalgesic (Mann–Whitney U-test, $P < 0.01$; Panels 1A and B). Immediately after the 3-h acute restraint session (Panel 1A), an increase in the TFL was observed in the control group (Wilcoxon Matched-Pairs test, $P < 0.01$), whilst no effect was observed in chronically-stressed animals (Wilcoxon Matched-Pairs test, $P > 0.05$). Exposure to forced swimming caused an antinociceptive effect in both groups (Panel 1B) (Wilcoxon Matched-Pairs test, $P < 0.002$ for the control group, and $P < 0.001$ for the chronically-stressed group).

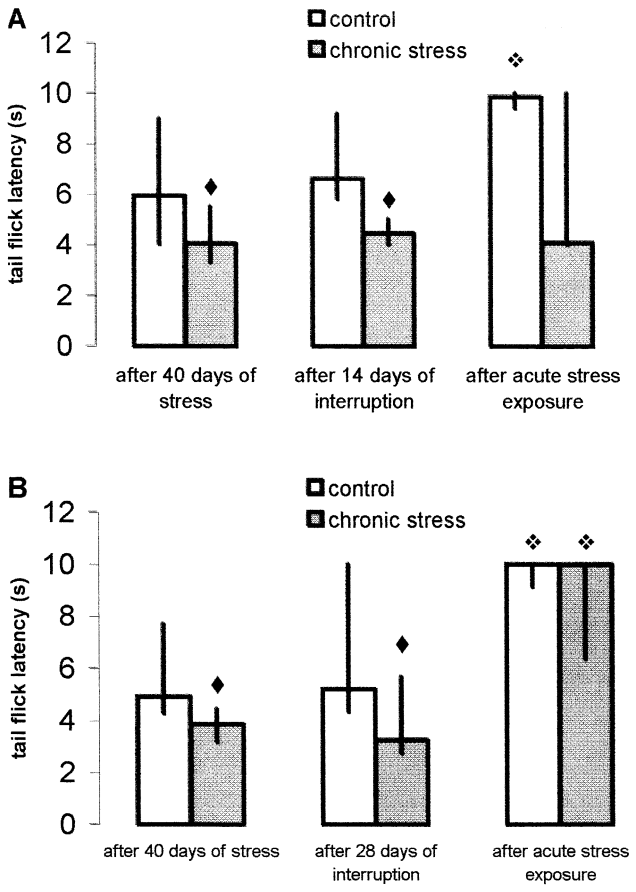

Fig. 1. Nociceptive response to the exposure to a 3 h restraint session after 14 days (Panel A), or 28 days (Panel B) after chronic stress interruption. The data are expressed as median (interquartile range) ($n = 7$–8/group). (♦) Significant difference compared to the measurement of the control group obtained at the same period (Mann–Whitney U-Test, $P < 0.05$. (❖) Significant difference between pre and post acute stress exposure (Wilcoxon Matched-Pairs test, $P < 0.01$).

4.2. Effect of a new restraint session upon the nociceptive response after 14- or 28-days of chronic stress interruption

Results are shown in Fig. 2. The animals presented decreased TFL after 40 days of treatment, and this effect persisted after the suspension of the stressor for either 14 or 28 days (Mann–Whitney U-test, $P < 0.05$; Panels 2A and B).

Fourteen days after the interruption of the treatment, the exposure to a 3 h restraint session did not affect the TFL in the stressed group (Fig. 2A; Wilcoxon Matched-Pairs test, $P > 0.05$); whilst the control group demonstrated increased latency, characterizing the classic SIA (Fig. 2A; Wilcoxon Matched-Pairs test, $P < 0.05$). After 28 days of chronic stress interruption, both groups presented an antinociceptive response to the exposure to a 3-h acute restraint session (Fig. 2B, Wilcoxon Matched-Pairs test, $P < 0.05$).

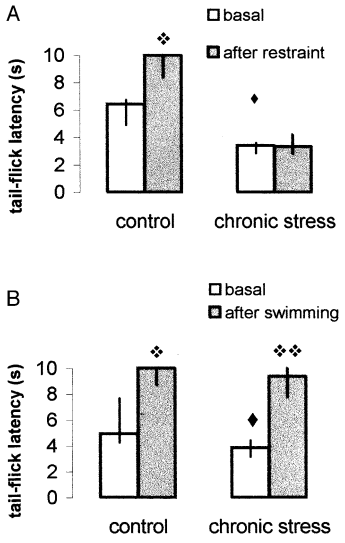

Fig. 2. Nociceptive response before (basal) and after exposure to one session of acute stress (*Panel A* —restraint for 3 h ($n = 10$ –15/group); *Panel B* —forced swimming (12–15/group)) in rats previously submitted to chronic stress. The data are expressed as median (interquartile range). (♦) Significant difference compared to the basal measurement of the control group (Mann–Whitney U-test, $P < 0.02$). (❖) Significant difference between basal and after acute stress latencies, Wilcoxon Matched-Pairs test, $P < 0.005$ for control group. (❖❖) Significant difference between basal and after acute stress latencies $P < 0.001$ for the chronically-stressed group.

4.3. Effect of morphine on nociception in repeatedly-stressed and control rats

Results referring to the analgesic effect of morphine are shown in Fig. 3.

Morphine administration increased the TFL in the control group. Morphine 1 mg/kg increased the TFL both 30 and 60 min after the administration (Friedmann test, $P < 0.05$ and $P < 0.02$, respectively), and morphine 5 mg/kg also had this effect ($P < 0.02$ for both times). In the stressed group, morphine had an effect only at the dose of 5 mg/kg ($P < 0.05$ at 30 min and $P < 0.02$ at 60 min; Friedmann test) when compared to the saline group.

5. Discussion

In the present study, the hyperalgesia induced by the repeated restraint stress procedure was maintained for 28 days after suspension of the treatment. The analgesic response to acute restraint stress was re-established only in this period and was not observed 14 days after the suspension. Data suggest that, in this model of chronic stress, the re-establishment of the activity of the systems involved in the nociceptive response have different patterns, according to the period of time and the response evaluated (the basal measurement or the nociceptive response to the acute stress). Prolonged

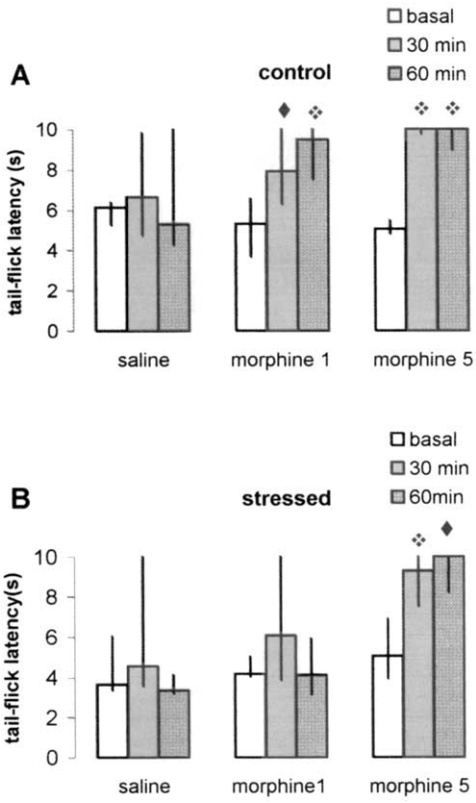

Fig. 3. Nociceptive response to morphine (1 or 5 mg/kg, i.p.) or saline after 40 days chronic restraint stress. *Panel A:* control group ($n = 8$–13/group); *Panel B:* stressed group ($n = 8$–10/group). The data are expressed as median (interquartile range). A Friedman ANOVA test shows effect of morphine 1 and 5 mg for the control group, and just of morphine 5 mg for the stressed group. (♦) Significant difference compared to the latency of this group obtained prior to morphine administration (Wilcoxon test, $P < 0.05$). (❖❖) Significant difference compared to the latency of this group obtained prior to morphine administration (Wilcoxon, $P < 0.02$).

stress could lead to more lasting alterations in the neural systems involved in nociception modulation, which persist even after the suspension of stress.

The mechanisms underlying long-lasting hyperalgesia after stress are poorly understood. Satoh et al. (1992) suggested that the long-lasting mechanical hyperalgesia (3 days) induced by prolonged cold stress involves peptide-containing primary afferents (substance-P and calcitonin-gene-related peptide). Quintero et al. (2000) suggested that the increased thermal and chemical nociception (8–9 days) observed after sub-chronic swimming stress might be mediated by changes in the activity of the central serotoninergic system. Work in humans shows that a reduction in the pain threshold after long-term psychoemotional stress was probably due to a reduction in the activity of the brain's opioid system (Ashkinazi and Vershinina, 1999). Previous data from our group also suggest the involvement of opioid systems in the hyperalgesic response induced by prolonged restraint stress (Gamaro et al., 1998; Torres et

al., 2001b). In the present study, hyperalgesia lasted for at least 28 days, which is longer than any of the works cited above. This is the first report of such a long hyperalgesia in animals after suspension of stress treatment.

We also evaluated whether chronic restraint animals could present SIA immediately after being submitted to acute stress. Different responses were observed, depending on the stressor used. When restraint (i.e., the same stressor used repeatedly for the chronic treatment) was utilized, the animals from the chronic stress group did not present any response to it, whilst control animals presented the classical antinociceptive effect. This was probably due to an adaptation of the stressed animals to this stressor. Conversely, forced swimming induced antinociception in both groups. This result suggested that chronically-stressed animals were able to respond to stress with an analgesic response, although they did not respond similarly to different stressors. Siegel (1977) suggested that animals tested in new context show analgesic response, while rats tested in the original context continue to exhibit tolerance to analgesia. Blustein et al., (1995) reported that the contextually mediated tolerance to forms of SIA engendered by repeated exposure to stimulus might be the consequence of repeated activation of endogenous opiates.

Concerning these results, previous works from the literature have suggested that, when animals are repeatedly submitted to the same stressor, some behavioral and physiological consequences of stress exposure are reduced (habituation). For example, ACTH or corticosterone levels are reduced after repeated exposure to the same (homotypic) stressor (Marti and Armario, 1998; Torres et al., 2001a), although negative results have been reported (Dal-Zotto et al., 2000). On the other hand, the response to a new stressor may be intact (Marti and Armario, 1998), or potentiated, a phenomenon termed facilitation (Marti et al., 1999; Garcia et al., 2000).

Years of research into the phenomenon of SIA have demonstrated the existence of multiple pain inhibitory systems (Lewis et al., 1982; Bodnar and Sikorszky, 1983; Rochford and Stewart, 1987). Analgesic response to stress has been typically characterized in terms of its mediation by opioid and non-opioid mechanisms (Lewis et al., 1980, 1982; Bodnar, 1986; Rochford and Stewart, 1987). That is, distinct neurochemical pain inhibitory mechanisms can be activated, depending on the properties of the stressor (e.g., severity, duration) (Lewis et al., 1980; Terman et al., 1986; Mogil et al., 1996) and on other factors (animal strain and gender) (Urca et al., 1985; Gamaro et al., 1998). Classically stress induces analgesic response with opioid participation (Pohorecky et al., 1999). However, in studies of swim stress-induced analgesia (SSIA) with mice and rats, the manipulation of parameters such as water temperature and swimming duration seems to modify the severity of stress and the role of different pain inhibitory pathways that are responsible for SIA, inducing opioid or non-opioid forms of SIA (Mogil et al., 1996; Hopkins et al., 1998; Omiya et al., 2000). It is possible that the analgesia elicited by forced swimming observed in our study is one form of non-opioid SIA. Since it is possible that chronic restraint stress could alter central opioid activity, and the next experiment will further investigate this possibility, non-opioid-induced analgesic response to swimming could be maintained.

In the last experiment, we tested control and repeatedly restrained rats injected with morphine (1.0 and 5.0 mg/kg). Our results demonstrate that repeatedly stressed rats display decreased morphine effects on nociception compared to unstressed controls. The stressed group needed a increased dose and longer time to show the classic analgesic effect of morphine. This change in sensitivity to morphine may be the result of alterations in treatment-induced peptides release, i.e., persistent activation of opiate peptide receptors by endogenous opioids released during restraint stress could lead to receptor down-regulation, but it is possible that interactions with other released neurotransmitter could induce these effects, for example, serotonin, glutamate, adenosine and other opioid receptor systems have also been involved.

Several studies have determined that analgesia induced by morphine is potentiated by acute stress or induced by opioids administered either systemically (Amir and Amit, 1978; Dilsaver et al., 1986; Cancela et al. 1988 Cancela et al. 1995) or intracerebroventricularly (Appelbaum and Holtzman, 1985; Bodnar 1986) in rats. In their reports, morphine-treated rats exposed to restraint stress showed a potentiated magnitude and duration of analgesia compared to unstressed rats, and consistent potentiation of analgesia in restrained rats was displayed by animals that were given agonists with high intrinsic activity at the mu (μ) receptor (Calcagnetti et al., 1990). This potentiation of the analgesia induced by opioids was higher in subjects that were first exposed to restraint stress, compared to habituated animals submitted to 5 days of 1 h restraint stress (Calcagnetti and Holtzman, 1991). Most of the reports from these investigators, however, were done with acute stress. In this study, the effects of morphine on nociception were not potentiated by exposure to stress, on the contrary, chronically treated rats displayed decreased morphine effects on nociception.

In addition, previous reports using rodents stressed chronically have reported the analgesic effect of morphine to be either enhanced (Lewis et al., 1981) reduced (Watkins et al., 1982; Girardot and Holloway, 1984) or unchanged (Bodnar et al., 1978; Girardot and Holloway, 1984) as compared to controls. These discrepancies are related to the nature of the stressor and to its pattern. The tolerance of response to morphine observed

in the present study agrees with the hypothesis suggested by previous studies that chronic restraint stress could modify the activity of opioid systems (for review, see Drolet et al., 2001). Changes in the sensitivity to opioid-induced antinociception observed in these rats might be due to alterations in central or peripheral opioid receptors, both in their affinity or number, or these changes might be due to alterations in other neurotransmitter or hormonal systems able to interact with these opioid receptors. In our laboratory, using this stress model, we observed decrease opioid receptors density in central nervous system (Dantas et al., personal communication). Since morphine exerts its antinociceptive effects primarily through μ opiate receptor subtype, the altered responses observed in animals submitted to repeated restraint stress might be due to changes at the level of these receptors.

In conclusion, the present experiments indicate that the hyperalgesia induced by repeated stress in rats persists for at least 28 days after suspension of the chronic treatment. When the animals were submitted to a new session of acute stress at the end of chronic restraint treatment, antinociception was observed with the exposition to a different stressor agent (forced swimming). The analgesic response to restraint stress was re-established only after 28 days of stress interruption, although the animals continued to present a decreased basal nociceptive threshold. Therefore, the mechanisms related to the long-lasting delayed hyperalgesia presented in this study are not exactly the same as those responsible for the analgesia induced by acute stressors. These differences have not been elucidated. It is possible that this long-lasting hyperalgesia is the result of a long-lasting tolerance of the HPA axis and/or alteration in opioid receptors and/or alteration in some other system related to the stress response, that likely involve some kind of learning-like plasticity. Which effect is the responsible for the displayed hyperalgesia and which is the responsible for the habituation to the stressor, whose effect on nociception is eventually reestablished, deserve further investigation. We also demonstrate that repeatedly-stressed rats showed tolerance to the morphine antinociceptive effect. Future studies should evaluate the activity of the opioid receptors in this model. Continued studies concerning the mechanisms of stress-induced hyperalgesia may be relevant to the research of the etiology of chronic pain disorders.

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CAPÍTULO II

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Effect of drugs active at adenosine receptors upon chronic stress-induced hyperalgesia in rats

Iraci Lucena da Silva Torres[a,*], Carla Denise Bonan[a,b], Leonardo Crema[a], Marcele De Leon Nunes[a], Ana Maria Oliveira Battastini[a], João José Freitas Sarkis[a], Carla Dalmaz[a], Maria Beatriz C. Ferreira[a,c]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Departamento de Ciências Fisiológicas, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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Abstract

Hyperalgesia and altered activities of enzymes involved in nucleotide hydrolysis are observed after exposure to repeated restraint in rats. Here, we investigated the effect of an adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA, 3.35 mg/kg, i.p.), adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.8 mg/kg, i.p.) as well the effect of an adenosine reuptake blocker, dipyridamole (5 mg/kg, i.p.), on nociception in chronically stressed and control rats. We repeatedly submitted rats to restraint for 40 days. Nociception was assessed with a tail-flick apparatus. The control group presented increased tail-flick latencies after administration of CPA and dipyridamole, but this effect was not observed in the stressed group. DPCPX by itself had no effect on nociception. The analgesic effect of CPA and dipyridamole observed in the control group was reverted by DPCPX. These results indicate the involvement of adenosine A_1 receptor in the antinociception observed in control animals and suggest that the pain signaling induced by chronic stress presents a different modulation involving the adenosinergic system.

Keywords: Hyperalgesia; Chronic stress; Adenosine; CPA; N6-cyclopentyladenosine; Dipyridamole; Tail-flick

1. Introduction

Acute exposure to a variety of stressors produces immediate analgesia in several pain tests (Watkins and Mayer, 1986; Bodnar, 1986). In addition, some studies have reported that, under some experimental conditions, both acute and chronic stress can elicit hyperalgesia instead of analgesia (Satoh et al., 1992; Quintero et al., 2000). Previous data from our laboratory showed decreased pain thresholds after exposure to repeated restraint stress in male rats (Gamaro et al., 1998; Torres et al., 2001a). Unlike stress-induced analgesia, the mechanisms involved in the stress-induced hyperalgesia are less known.

Adenosine is one of the main neuromodulators associated with cell stress (Cunha, 2001). Indeed, it is known that expression of adenosine A_1 receptors is increased by glucocorticoids (Svenningsson and Fredholm, 1997). Increase in extracellular adenosine concentration has been observed following stressful challenges (Latini and Pedata, 2001), including exposure to inescapable shock (Minor et al., 2001). Extracellular adenosine can be released as such through bidirectional non-concentrative adenosine transporters (Cass et al., 1998) or can originate from the extracellular catabolism of released ATP through the ecto-nucleotidase pathway (Cunha, 2001; Zimmermann, 1996). ATPase, ADPase and 5′ -nucleotidase activities in the blood serum were increased by acute restraint stress (Böhmer et al., in press). A previous work showed that chronically stressed male rats exhibit a decreased ADP hydrolysis in synaptosomes from the spinal cord (Torres

* Corresponding author. Departamento de Farmacologia-ICBS, UFRGS, Rua Sarmento Leite, 500 sala 202, 90050-170 Porto Alegre, RS, Brazil. Tel.: +55-51-3316-3183; fax: +55-51-3316-3121.
E-mail addresses: torresf@terra.com.br (I.L. da Silva Torres), mariabea@vortex.ufrgs.br (M.B.C. Ferreira).

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et al., 2002a), with no effect when acute stress was utilized. Additionally, we also showed that only hydrolysis of ADP was altered in the blood serum of chronically stressed male rats (Torres et al., 2002b). In contrast, when the animals were submitted to acute stress, opposite effect was observed, i.e., an increase in the nucleotidase pathway in rat serum (Böhmer et al., in press), which agrees with previous studies using acute stress models (see reviews of Cunha, 2001; Latini and Pedata, 2001). Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single stress episode (Hashiguchi et al., 1997; Torres et al., 2001b), and the phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes, including those regulating adenosine availability.

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the surface, or may be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). Members of several families of ecto-nucleotidases can contribute to the extracellular hydrolysis of nucleotides. Nucleoside 5′-tri- and diphosphates (NTP and NDP) may be hydrolyzed by members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP) family and by alkaline phosphatases (Zimmermann, 2001). These ecto-nucleotidases, together with 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this cascade formed by ecto-nucleotidases and 5′-nucleotidase is an enzymatic pathway with a double function of removing a signal of ATP and generating a second one, adenosine. These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological conditions (Agteresch et al., 1999).

It has been proposed that extracellular adenosine is involved in physiological pain control at the spinal cord level and in opioid antinociception (Sawynok and Liu, 2003). Animal studies have demonstrated adenosine-mediated inhibitory influences on presumed nociceptive reflex responses (Sawynok, 1998), possibly through the adenosine A_1 receptors (Keil and DeLander, 1996). Adenosine analogs have antinociceptive properties in experimental and clinical situations, including neuropathic pain, where pain-signaling mechanisms have been altered (Sawynok, 1998; Jarvis and Kowaluk, 2001).

In this study, we investigated the effect of administration of an adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA) and antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), as well the blockade of adenosine uptake with dypiridamole on chronic stress-induced hyperalgesia in rats, to explore the role of adenosine in this process.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (200–230 g) from our breeding stock were used. Experimentally naive animals were housed in groups of five in home cages. They were maintained on a standard 12-h dark/light cycle (lights on 7:00 a.m.) at room temperature (22 ± 2 °C). The rats had free access to food and water, except during the period of exposure to the stressor. The Institutional Research Committee approved all animal procedures, and measures were taken to minimize pain and discomfort.

2.2. Chronic restraint stress procedure

The animals were stressed by restraint 1 h daily, 5 days/week for 40 days (Ely et al., 1997). Restraint was carried out by placing the animal in a 25 × 7-cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1-cm hole in the far end for breathing. The control group was not submitted to stress and the animals were kept in their home cages. The immobilization procedure was always performed between 10:00 a.m. and 1:00 p.m.

2.3. Tail-flick measurement

Nociception was assessed with the tail-flick apparatus (D'Amour and Smith, 1941). Rats were wrapped in a towel and placed on the apparatus. The light source positioned below the tail was focused on a point 2–3 cm rostral at the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. The tail-flick latency represented the period of time (s) from the beginning of the trial to the tail deflection. Light intensity was adjusted so to obtain baseline tail-flick latencies of 3–4 s (0.7 mA). A cut-off time of 10 s was used to prevent tissue damage. After the last session of the treatment (40 days) and 24 h before injection of the drugs, the animals were exposed to the tail-flick apparatus to familiarize them with the procedure, since the novelty of the apparatus can itself induce antinociception (Netto et al., 1987).

2.4. Drugs administration

The drugs used were CPA (3.35 mg/kg), DPCPX (0.8 mg/kg) and dipyridamole (5 mg/kg). CPA was dissolved in 0.9% NaCl, DPCPX in 5% dymethyl sulfoxide + 1.25% NaOH 1 M and dipyridamole was dissolved in 0.9% NaCl, pH 4.0. All drugs were administered i.p. in a volume of 1.0 ml/kg, 24 h after last session of stress. Tail-flick latencies were measured before (basal measure), at 30 and 60 min after the injection, depending on the drug tested. The protocol of associated administration of

Table 1
Nociceptive response to CPA (3.35 mg/kg) or vehicle after chronic stress

Drugs	Time	Control group	Stressed group
	Basal measure (s)	4.22 ± 1.92 (22)	2.93 ± 1.18 (28)[a]
Vehicle	30 min (Δ)	− 0.80 ± 0.63 (11)	0.45 ± 0.14 (14)
	60 min (Δ)	− 1.67 ± 0.54 (11)	0.20 ± 0.23 (14)
CPA	30 min (Δ)	0.92 ± 1.14 (11)	0.05 ± 0.28 (14)
(3.35 mg/kg)	60 min (Δ)	2.72 ± 1.40[b] (11)	0.29 ± 0.39 (14)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].
[a] Significant difference compared to the control group (Student's *t*-test, $P = 0.009$).
[b] Significant difference compared to the latency of the respective vehicle group (Student's *t*-test, $P < 0.02$).

CPA or dipyridamole plus DPCPX consists of a first injection of CPA or dipyridamole, immediately followed by an injection of DPCPX, at similar doses previously tested.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. of Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)]. Basal measure was expressed in seconds. Statistical significance was determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

In all experiments using control and stressed animals, the basal measures were compared and this revealed a significant difference between the groups. The stressed group showed a hyperalgesic effect in all experiments (Student's *t*-test, $P < 0.05$; (Tables 1, 2 and 4)).

3.1. Effect of CPA and DPCPX on the tail-flick test

CPA (3.35 mg/kg, i.p.) produced a significant analgesic effect in the control group at 60-min group after the drug administration (Student's *t*-test, $P < 0.02$). CPA had no significant effect upon nociception at any time after the drug administration (30 and 60 min) in stressed rats (Student's *t*-test, $P > 0.05$). Results are presented in Table 1.

DPCPX (0.8 mg/kg), at 30 and 60 min, did not promote significant changes in the nociception in the tail-flick test for the control and stressed group (Student's *t*-test, $P > 0.05$; Table 2).

To confirm the involvement of adenosine A_1 receptors in the analgesic action of CPA, we tested the associated administration of CPA plus DPCPX in the control group. CPA plus DPCPX did not show effects in the tail-flick test, suggesting that DPCPX reverted the analgesic effect induced by CPA in the control rats (Student's *t*-test, $P > 0.05$; Table 3).

Table 2
Nociceptive response to DPCPX (0.8 mg/kg) or saline after chronic stress

Drugs	Time	Control group	Stressed group
	Basal (s)	5.99 ± 1.75 (16)	4.15 ± 1.21[a] (14)
Vehicle	30 min (Δ)	0.57 ± 0.43 (10)	3.42 ± 1.15 (6)
	60 min (Δ)	0.41 ± 0.58 (10)	0.20 ± 0.23 (6)
DPCPX	30 min (Δ)	0.26 ± 0.97 (6)	0.05 ± 0.28 (8)
(0.8 mg/kg)	60 min (Δ)	0.40 ± 0.60 (6)	0.29 ± 0.39 (8)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].
[a] Significant difference compared to the control group (Student's *t*-test, $P = 0.003$).

Table 3
Nociceptive response to CPA (3.35 mg/kg) + DPCPX (0.8 mg/kg) or saline + vehicle in control group

Drugs	Time	Control Group
Saline + vehicle	30 min (Δ)	0.03 ± 0.61 (8)
	60 min (Δ)	0.78 ± 0.77 (8)
CPA (3.35 mg/kg) +	30 min (Δ)	0.52 ± 0.59 (10)
DPCPX (0.8 mg/kg)	60 min (Δ)	− 0.57 ± 0.74 (10)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].

3.2. Effect of dipyridamole on the tail-flick test

Administration of dipyridamole (5 mg/kg, i.p.) produced an analgesic effect at 30 min (Student's *t*-test, $P < 0.0001$) and 60 min (Student's *t*-test, $P < 0.05$) after drug administration in the control group. Dipyridamole had no significant effect upon nociception at any time after drug administration (30 and 60 min) in stressed rats (Student's *t*-test, $P > 0.05$). Results are presented in Table 4.

To confirm the involvement of adenosine in the analgesic action of dipyridamole, we tested the associated administration of dipyridamole plus DPCPX in the control group. Dipyridamole plus DPCPX did not show effects in the tail-flick test, suggesting that DPCPX reverted the analgesic

Table 4
Nociceptive response to dipyridamole (5 mg/kg) or saline after chronic stress

Drugs	Time	Control	Stressed
	Basal (s)	4.54 ± 2.42 (22)	3.36 ± 1.46[a] (28)
Vehicle	30 min (Δ)	− 0.42 ± 0.43 (13)	0.71 ± 0.39 (13)
	60 min (Δ)	0.78 ± 0.43 (13)	1.24 ± 0.46 (13)
Dipyridamole	30 min (Δ)	3.25 ± 0.69[b] (9)	0.68 ± 0.43 (15)
(5 mg/kg)	60 min (Δ)	2.97 ± 0.90[b] (9)	0.69 ± 0.62 (15)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].
[a] Significant difference compared to the control group (Student's *t*-test, $P < 0.05$).
[b] Significant difference compared latency of the respective vehicle group (30 min: Student's *t*-test, $P = 0.0001$; 60 min: Student's *t*-test, $P < 0.05$).

Table 5
Nociceptive response to dipyridamole (5 mg/kg) + DPCPX (0.8 mg/kg) or saline + vehicle in control group

Drugs	Time	Control Group
Saline ± vehicle	30 min (Δ)	− 0.05 ± 0.52 (6)
	60 min (Δ)	− 0.47 ± 0.87 (6)
Dipyridamole (5 mg/kg) ± DPCPX (0.8 mg/kg)	30 min (Δ)	− 1.61 ± 0.7 (6)
	60 min (Δ)	− 0.70 ± 0.77 (6)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].

effect induced by dipyridamole in the control rats (Student's *t*-test, $P > 0.05$; Table 5).

4. Discussion

In this study, we observed an analgesic effect of CPA and dipyridamole in control animals, but no effect in chronically stressed rats. Additionally, the effect of CPA and the effect of dipyridamole on nociception in control rats were prevented by DPCPX, an antagonist of A_1 receptors, suggesting that the antinociception induced by CPA or dipyridamole is mediated through these receptors. The absence of effect observed on chronically stressed animals indicates a different modulation of pain signaling induced by chronic stress involving the adenosinergic system.

Previous studies have demonstrated that the manipulation of endogenous adenosine levels induces antinociception in the mouse tail-flick test (Keil and DeLander, 1994). The rat tail-flick test used in the present study involves a spinal nociceptive reflex, and it is thus suitable for studying the adenosinergic influence. In addition, i.t. administration of adenosine receptor antagonists induces thermal hyperalgesia in the tail-flick test under certain conditions (Sawynok et al., 1986). These results indicated that an endogenous purinergic system might be active at spinal sites modulating nociceptive neurotransmission. Facilitation of this system would be expected to induce antinociception, whereas its inhibition would result in facilitated nociceptive neurotransmission.

Chronically stressed male rats exhibit a decreased tail-flick latency just after an exposure to restraint, indicating a hyperalgesic response (Gamaro et al., 1998). Spinal systems, including opioid and adenosine purinergic systems, modulate nociceptive neurotransmission in the dorsal horn (Yaksh and Malmberg, 1994). Furthermore, evidence indicates that chronic restraint stress induces a decrease in the sensitivity to morphine (Torres et al., 2003). Several studies support the hypothesis that adenosine is involved in opioid-induced antinociception (Sawynok and Liu, 2003). In this study, whilst CPA, an adenosine A_1 receptor agonist, induced an analgesic response in the control group, no effect was observed when it was administrated in chronically stressed animals. It is important to consider that the drugs tested were administered intraperitoneally, being possible a partial contribution of peripheral adenosine A_1 receptors in the analgesic effects observed. Our results suggest that alterations in adenosine A_1 receptors might be involved in the hyperalgesia observed in stressed rats.

Nucleoside transport process may play a role in regulating endogenous levels of the adenosine in the central nervous system. Equilibrative nucleoside transporters (ENT) carry nucleosides across cell membranes in either direction according to their concentration gradients. Two ENT subtypes accepting both purine and pyrimidine nucleosides as well as a number of synthetic nucleoside analogs have been cloned and termed ENT1 and ENT2. ENT1 and ENT2, and present a wide cellular and regional distribution in rat and human brain (Anderson et al., 1999a,b). ENT1 is differentiated from ENT2 by its sensitivity to inhibition by nanomolar concentrations of the nucleoside analog nitrobenzylthioinosine, but both are inhibited by dipyridamole (Cass et al., 1998). The nucleoside transporter inhibition can significantly increase basal extracellular adenosine concentrations, probably due to inhibition of nucleotide-derived adenosine reuptake. Inhibition of this uptake could cause greater synaptic adenosine levels and subsequently increased activation of extracellular adenosine receptors, and these effects can induce antinociception (Sweeney et al., 1993). Furthermore, morphine has been demonstrated to release adenosine per se from primary afferent neurons, mediated by nucleoside transporters, sensitive to the inhibitor dipyridamole but insensitive to nitrobenzylthioinosine (Sweeney et al., 1993). In agreement with previous studies (Zarrindast et al., 1993), the administration of nucleoside transport inhibitors induced an antinociceptive effect in naive rats. On the other hand, this effect was not observed in stressed rats. Extracellular ADP hydrolysis has been demonstrated to decrease in stressed rats (Torres et al., 2002a,b), which could induce decreased levels of extracellular adenosine. According to the present results, inhibition of adenosine reuptake by dipyridamole in stressed rats is not enough to compensate this possible reduction of extracellular adenosine levels. Previous studies from our laboratory have demonstrated that acute stress induces an increase in the nucleotidase pathway in rat serum (Böhmer et al., in press). Repetitive exposure to restraint stress could induce an adaptative response in chronically stressed animals, which could lead to a desensitization of adenosine receptors. Previous work showed that mice lacking the adenosine A_1 receptor showed hyperalgesic responses (Johansson et al., 2001). Therefore, the lack of effect of the drugs tested in the stressed animals can be due to: (1) decreased effectiveness of adenosine A_1 receptors or (2) stress-induced augmentation of the extracellular levels of adenosine that would saturate adenosine A_1 receptors (Cunha, 2001; Latini and Pedata, 2001). These results further support the hypothesis that adenosinergic modulation is altered in chronically stressed animals.

In summary, we demonstrated an absence of the adenosine antinociceptive effect in chronically stressed rats. Future

studies concerning the mechanisms of stress-induced hyperalgesia may be relevant for the research into the etiology of chronic pain disorders.

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CAPÍTULO III

ELSEVIER

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PHYSIOLOGY & BEHAVIOR

Effect of chronic and acute stress on ectonucleotidase activities in spinal cord

Iraci Lucena S. Torres[a,*], Andréia Buffon[a], Patricia Pellufo Silveira[a], Marcelo Z.D. Duarte[a], Marcio Garcia Bassani[a], Silvana S. Oliveira[a], Ana Maria O. Battastini[a], João J.F. Sarkis[a], Carla Dalmaz[a], Maria Beatriz C. Ferreira[b]

[a]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, 90035-003, Porto Alegre, RS, Brazil*
[b]*Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, UFRGS, 90035-003, Porto Alegre, RS, Brazil*

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Abstract

We have previously observed that, while acute stress induces analgesia, chronic stress causes a hyperalgesic response in male rats. No effect was observed in females. There is increasing evidence that both ATP and adenosine can modulate pain. Extracellular ATP and ADP are hydrolyzed by an apyrase in synaptosomes from the peripheral and central nervous systems. In the present study, we investigated the effect of chronic and acute stress on ATPase–ADPase and 5′-nucleotidase activities in spinal cord of male and female rats. Adult male and female Wistar rats were submitted to 1 h restraint stress/day for 1 day (acute) or 40 days (chronic) and were sacrificed 24 h later. ATPase–ADPase activities were assayed in the synaptosomal fraction obtained from the spinal cord of control and stressed animals. ADP hydrolysis was decreased 25% in chronically stressed males, while no change was observed on ATPase activity. There was an increase in the 5′-nucleotidase activity in the same group. No effect on ADPase, ATPase or on 5′-nucleotidase activity was observed in females with chronic stress, or after acute stress neither in males or females. Chronic stress reduced ADP hydrolysis and increased 5′-nucleotidase activity in the spinal cord in male rats.

Keywords: Stress; ATPase–ADPase activities; 5′-nucleotidase activity; Spinal cord; Nociception

1. Introduction

It is well known that individuals exposed to acute stressful conditions show an increase in pain threshold, called stress-induced analgesia (SIA) [2,4,25]. A previous study [22] showed that animals submitted to repeated restraint stress presented adaptation that varied according to the gender. Chronically stressed male rats showed a decrease in tail-flick latency both in the basal state and just after exposure to restraint. Chronically stressed females did not respond to the stress session with the same hyperalgesic effect.

Adenosine 5′-triphosphate (ATP) is a purine nucleotide found in every cell. In addition to its well-established role in cellular metabolism, extracellular ATP and its breakdown product adenosine have pronounced effects in a variety of biological processes, including neurotransmission [1,13,32]. There is increasing evidence that adenosine and ATP may act as pain neuromodulators in the spinal cord [35,36,47]. Intrathecal administration of P_2-purinoceptor antagonists has been associated with an antinociceptive action [19,47,48]. In addition, there are reports that ATP acts on dorsal horn neurons in the spinal cord after being released from a subpopulation of small primary afferent nerves involved in pain pathways [21,41], and the purinergic cascade in nociception is recently reviewed by Jarvis and Kowaluk [27].

The release of adenosine appears to have functional importance with respect to regulating nociceptive thresholds [28,42] and mediating a component of spinal analgesia by morphine and 5-HT [17,18]. A cascade of ectonucleotidases may play a role in the effective regulation of these processes and may also have a protective function by keeping extracellular ATP and adenosine levels within physiological

* Corresponding author. Departamento de Bioquímica, ICBS, UFRGS, Ramiro Barcelos, 2600, Anexo, Lab. 32, 90035-003 Porto Alegre, RS, Brazil. Tel.: +55-51-316-5577; fax: +55-51-316-5540.

E-mail address: torresf@zaz.com.br (I.L.S. Torres).

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levels [1]. ATP as well as other nucleotide agonists for P_2-puriceptors could be degraded by ectonucleotidases [51].

It has been reported that extracellular ATP is hydrolyzed by an ATP diphosphohydrolase (apyrase, ATPDase, EC 3.6.1.5) in synaptosomes of the peripheral and central nervous systems [3,37–39,43], splitting off the two phosphate groups, and generating AMP, which is later hydrolyzed by a 5′-nucleotidase (EC 3.1.3.5) yielding adenosine. The enzyme apyrase is able to hydrolyze all nucleotides, di- and triphosphates. A role for apyrase activity in neurotransmission has been suggested [5,50,51], and alterations in this enzyme activity appear to be related to some brain processes like memory acquisition and epilepsy [5–7]. Apyrase, together with 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation [11].

In the present study, we investigated the effect of chronic and acute restraint stress in the ATPase–ADPase and 5′-nucleotidase activities in spinal cord of adult male and female Wistar rats.

2. Materials and methods

2.1. Subjects

Adult male and female Wistar rats (60 days at the beginning of the treatment, weighing 150–230 g) were used. Experimentally naive animals were housed in groups of five in home cages made of Plexiglas material ($65 \times 25 \times 15$ cm) with the floor covered with sawdust and maintained on a standard dark–light cycle (lights on between 7 a.m. and 7 p.m.) at a room temperature of 22 ± 2 °C. The rats had free access to food (standard lab rat chow) and water, except during the period when restraint stress was applied. The immobilization procedure was performed between 10 and 12 a.m.

2.2. Chronic restraint stress procedure

The animals were divided into two groups: stressed and control. Restraint was applied by placing the animals in a 25×7 cm plastic bottle, and fixing it with plaster tape on the outside so that the animal was unable to move. There was a 1-cm hole at one far end for breathing. The animals were stressed 1 h/day, 5 days a week for 45 days [20]. Control animals were kept in their home cages.

2.3. Acute procedure

The animals were divided into two groups: stressed and control. Restraint was applied using the same procedure as described above. The animals were stressed for 1 h, 24 h before the assay. Control animals were kept in their home cage.

2.4. Subcellular fractionation

Approximately 24 h after the last stress session (chronic) or the only session (acute), the animals were killed by decapitation and the spinal cord was rapidly removed and gently homogenized in 10 vols. of ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, with a motor driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously [33]. Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4 ml 8.5% Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10/20%). The synaptosomes that banded at the 10/20% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

2.5. Enzyme assays

The reaction medium used to assay ATPase–ADPase activities was described previously [3]. The medium contained 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The synaptosomal fraction (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 μl samples were taken for the assay of released inorganic phosphate (Pi) [10].

Table 1
Effect of acute stress on apyrase activity (ATP and ADP hydrolysis), on the ATPase–ADPase ratio and on 5′-nucleotidase activity in synaptosomes from spinal cord of male ($n = 6–8$ animals/group) and female rats ($n = 8$ animals/group)

	Males		Females	
	Control group	Stressed group	Control group	Stressed group
ATPase	181.20 ± 14.23	200.50 ± 8.84	189.49 ± 21.68	228.82 ± 12.32
ADPase	52.79 ± 5.45	49.16 ± 4.43	38.97 ± 6.27	41.13 ± 3.44
ATPase–ADPase	3.55 ± 0.32	4.19 ± 0.30	5.59 ± 0.77	5.88 ± 0.63
Ecto 5′-nucleotidase	27.01 ± 3.05	21.54 ± 2.61	21.01 ± 3.52	19.04 ± 1.43

Values are mean ± S.E.M. of specific activity (pmol phosphate production/mg protein). There were no differences between stressed and control groups (Student's t test, $P > .05$).

Table 2
Effect of chronic stress on apyrase activity (ATP and ADP hydrolysis), on the ATPase–ADPase ratio and on 5′-nucleotidase activity in synaptosomes from spinal cord of male ($n=6–7$ animals/group) and female rats ($n=4–10$ animals/group)

	Males		Females	
	Control group	Stressed group	Control group	Stressed group
ATPase	265.55 ± 29.46	240.53 ± 17.93	129.45 ± 12.24	129.87 ± 7.74
ADPase	59.40 ± 4.51	44.06 ± 3.22[a]	27.22 ± 2.44	33.09 ± 2.19
ATPase–ADPase	4.44 ± 0.81	5.52 ± 0.92[b]	4.16 ± 0.34	4.85 ± 0.32
Ecto 5′-nucleotidase	18.39 ± 2.29	30.89 ± 4.19[b]	29.25 ± 3.87	22.91 ± 2.59

Values are mean ± S.E.M. of specific activity (pmol phosphate production/mg protein).
[a] Significant difference from control group (Student's t test, $P<.02$).
[b] Significant difference from control group (Student's t test, $P<.05$).

The reaction medium used to assay the AMP hydrolysis contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200 μl [26]. The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and stopped by the addition of 200 μl of 10% trichloroacetic acid; 100 μl of samples were taken for the assay of released inorganic phosphate (Pi) [10].

In both enzyme assays, incubation times and protein concentration were chosen in pilot studies to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in duplicate. Protein was measured by the Coomassie Blue method [8], using bovine serum albumin as standard.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. and analyzed by Student's t test.

3. Results

3.1. Effect of acute stress on ATPase–ADPase and 5′-nucleotidase activities in male and female rats

Both in male and female rats, the ATPase and ADPase activities as well as 5′-nucleotidase activity in synaptosomes from spinal cord did not show any change (Student's t test, $P>.05$) compared to controls after acute stress (Table 1).

3.2. Effect of chronic stress on ATPase–ADPase and 5′-nucleotidase activities in male and female rats

ADPase activity in synaptosomes from the spinal cord of male rats was significantly inhibited (25% in relation to controls) after chronic stress (Student's t test, $P<.02$), while no effect was observed in females. In addition, 5′-nucleotidase activity was increased in synaptosomes from chronically stressed male rats (Student's t test, $P<.05$), but no effect was observed in females. There was no alteration on ATPase activity in any case (Table 2).

4. Discussion

In the present experiments, we showed that ADPase and 5′-nucleotidase activities are altered in chronically stressed male rats. Chronically stressed male rats also exhibit a decreased tail-flick latency, characterizing a hyperalgesic response [22]. On the other hand, either acutely or chronically stressed female rats showed no effect of stress on any of these enzyme activities, and did not exhibit the hyperalgesic response observed in male rats [22].

It is important to observe that the effects of chronic stress on nociception are very different from the effects of acute stress on this parameter. While acute stress induces a transient increase in nociceptive threshold, chronic stress decreases the pain threshold with a long-lasting effect [22]. We observed that hyperalgesia is maintained even 28 days after the end of stress (data not shown). The mechanisms involved in the effects of chronic and acute stress on nociception are probably different, and alterations in enzyme activities may be important with respect to chronic stress.

Inhibition of ADPase activity, as observed in chronically stressed male rats, could result in a decrease in adenosine production from extracellular ATP breakdown. However, when measuring 5′-nucleotidase activity, it was observed that this enzyme activity is increased in chronically stressed male rats. Alterations were not observed in any other group. This may be a compensatory effect, due to a reduction in AMP production in the extracellular medium. The ATPase–ADPase ratio can have an important role on the signaling properties of ATP [11]. When apyrase is active, extracellular ATP is converted to AMP and then to adenosine by 5′-nucleotidase, and ADP is not an appreciable product. However, when apyrase is inhibited, as is the case in synaptosomes from chronically stressed males, ATP is converted to ADP by other ATPases, and ADP will be relatively stable. In this case, the AMP that is substrate to 5′-nucleotidase may be reduced. In addition, the reaction catalyzed by 5′-nucleotidase is the rate-lim-

iting step in this extracellular pathway from ATP to adenosine (for a review, see Ref. [14]). It is important to observe that this enzyme is inhibited by ATP and/or ADP [16]. So, only when ATP and ADP levels decrease below the threshold of inhibition of 5′-nucleotidase will adenosine be formed in an important amount. Since in the case of chronically stressed rats ADP may accumulate due to decreased ADPase activity, it is difficult to infer if the increased 5′-nucleotidase activity, which was measured in vitro, will or will not result in increased extracellular adenosine in vivo. Conversely, the ATPase activity did not change probably due to an up-regulation of an ecto-ATPase that is coexpressed with the ATP diphosphohydrolase in central nervous system [29].

The apparent dissociation observed between the two substrates (ATP and ADP) may be due to the simultaneous presence of at least two different enzymes involved in ATP hydrolysis, an ecto-ATP diphosphohydrolase and an ecto-ATPase [29,51], whereas only one enzyme, an ecto-ATP diphosphohydrolase, is involved in ADP hydrolysis. The presence of different populations of nucleotidases is also supported by the increased ATPase–ADPase ratio observed in synaptosomes from chronically stressed rats.

It has been proposed that endogenous adenosine formation is involved in physiological pain control at the spinal cord level and that its release is involved in the action of opioid antinociception [9,44,45]. Animal studies have demonstrated adenosine- and adenosine analog-mediated inhibitory influences on presumed nociceptive reflex responses [40,44]. Adenosine analogs have antinociceptive properties in a wide range of test systems, including those for neuropathic pain, where pain-signaling mechanisms have been altered [40]. On the other hand, there are reports showing no effect of intrathecal adenosine in relieving allodynia-like behavior [49]. In this sense, it is important to observe that the effects of purines on nociception are complex, depending on the subtype of receptor activated and on the localization where these substances are applied.

The adenosine receptors of the A_1-subtype are associated with a modulatory effect on pain transmission at the spinal cord level [30]. A_1 agonists appear to act presynaptically inhibiting the release of neurotransmitters or postsynaptically reducing neuronal excitability [24,31,34]. However, adenosine may also act on A_2 receptors causing increased neurotransmitter release [15,47]. The dorsal spinal cord contains both A_1 and A_2 adenosine receptors [12,23,41]. Adenosine modulation by A_1/A_2 receptors is present in different regions of the CNS, and different effects have been observed when distinct brain regions are considered [31,46].

It is also important to observe that in the acute and chronic stress experiments animals with different ages were used. In the acute stress experiments, animals used were 60 days old, while after chronic stress treatment, animals were 100 days old. Since different enzyme activities were observed in the control groups, it is possible that in this structure, these enzymes present alterations with the age, as well as with the sex of the animal.

Although the nature of pain modulation by adenosine is clearly complex, this receptor system is a potential target for the development of agents to control pain. The direct and indirect manipulation of the purinergic system may therefore yield novel therapies for pain control in altered pain states.

Further investigation is required to determine the effects of P_1 and P_2 receptors antagonists on the changes in nociception induced by chronic stress in male rats. The exact biochemical mechanism involved in the nociceptive effect after chronic stress, and if there is a relationship between nucleotide hydrolysis inhibition and the induction of hyperalgesia still deserves more detailed studies. It is also important to observe that most works concerning nociception/antinociception with ATP or adenosine were done using male rats. On the basis of the present results, gender based investigations are warranted.

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CAPÍTULO IV

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Chronic stress effects on adenine nucleotide hydrolysis in the blood serum and brain structures of rats

Iraci Lucena S. Torres[a,*], Andreia Buffon[a], Giovana Dantas[a], Cristina Ribas Fürstenau[a], Ana Elisa Böhmer[a], Ana Maria O. Battastini[a], João J.F. Sarkis[a], Carla Dalmaz[a], Maria Beatriz C. Ferreira[b]

[a]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Ramiro Barcelos, 2600 Anexo, Lab. 32, 90035-003 Porto Alegre, RS, Brazil*
[b]*Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, UFRGS, 90035-003, Porto Alegre, RS, Brazil*

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Abstract

We have previously observed that adenosine 5′-diphosphate (ADP) hydrolysis was decreased 25% in spinal cord synaptosomes of chronically stressed male rats, while no changes were observed in ATPase activity. In the present study, we investigated the effect of chronic stress on the hydrolysis of adenine nucleotides in two cerebral structures (frontal cortex and hypothalamus) and in the blood serum of male rats. Adult male Wistar rats were submitted to 1-h restraint stress/day for 45 days (chronic) and were sacrificed 24 h after the last session of stress. Adenosine 5′-triphosphate (ATP) or ADP hydrolysis was assayed in the synaptosomal fraction obtained from the frontal cortex and hypothalamus of control and chronically stressed animals. No effects on ADP or ATP hydrolysis were observed in any of the cerebral structures analyzed after chronic stress. On the other hand, reduced ADP hydrolysis was observed in the blood serum of chronic stressed rats. It is possible that the effects observed in the blood serum may represent an adaptation to chronic stress and may reflect different functions of nucleotides and/or enzymes in these tissues. It is possible that altered levels of ADPase activity in the serum may be a biochemical marker for chronic stress situations.

Keywords: Apyrase; Chronic stress; Nucleotide hydrolysis; Rat blood serum; Hypothalamus; Cerebral cortex

1. Introduction

Adenosine 5′-triphosphate (ATP) is a purine nucleotide found in millimolar concentrations in virtually all cells. In addition to its well-established role in cellular metabolism, extracellular ATP and its breakdown products, adenosine diphosphate (ADP) and adenosine, have pronounced effects in a variety of biological processes, including neurotransmission, muscle contraction, cardiac and platelet function, vasodilatation and liver glycogen metabolism (Agteresch et al., 1999).

ATP is recognized as a neurotransmitter in sympathetic, parasympathetic and sensory nerves in the periphery, as well as in the central nervous system (CNS) (Edwards et al., 1992). The receptors for ATP, P2-puriceptors, have been described in virtually every major organ and/or tissue system that has been studied (Burnstock and Williams, 2000). Apart from neuronal release as a transmitter or a cotransmitter, there are several other sources of extracellular ATP. ATP is a ubiquitous intracellular constituent, and, therefore, any cell could potentially provide it. However, the more likely role of released ATP may be to act as a neurotransmitter in both peripheral and central neurons (for a review of ATP, see Cunha and Ribeiro, 2000).

Besides ATP, its breakdown product adenosine has also several functions within the CNS, which involve an inhibitory tone of neurotransmission and neuroprotective actions in pathological conditions (Latini and Pedata, 2001). Adenosine is particularly well suited to be used as a transcellular messenger to signal metabolic imbalance. Several reports have documented an increased in the extracellular concen-

* Corresponding author. Tel.: +55-51-3316-5577; fax: +55-51-3316-5540.
E-mail address: torresf@zaz.com.br (I.L.S. Torres).

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tration of adenosine upon stressful metabolic challenges (Latini and Pedata, 2001), and the term "retaliatory metabolite" has been coined for this homeostatic role of adenosine, which occurs in virtually all cell types. (For a review on adenosine, see Cunha, 2001.)

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the cell surface, or may be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). In addition, soluble nucleotidases, which also break down ATP and adenosine, have also been shown to be released from sympathetic nerves (Todorov et al., 1997). Work of the past few years has demonstrated that members of several families of ectonucleotidases can contribute to the extracellular hydrolysis of nucleotides. Nucleoside 5′triphosphate (NTP) and nucleoside 5′diphosphate (NDP) may be hydrolyzed by members of the E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) family, E-NPP (ectonucleoside pyrophosphatase/phosphodiesterase) family and by alkaline phophatases (Zimmermann, 2001). These ectonucleotidases, together with 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors, and, consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this cascade of ectonucleotidases is an enzymatic pathway with a double function of removing a signal (ATP) and generating a second one (adenosine). This cascade may play a role in the effective regulation of several processes, because they have considerable plasticity in different pathophysiological situations (Agteresch et al., 1999) including aging (Cunha et al., 2001). These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological levels (Agteresch et al., 1999).

The physiological response to emotional or physical stress consists of an integration of endocrine and autonomic changes. In this situation, adrenomedullary epinephrine is released, and hormones such as CRH, ACTH and glucocorticoids are released by the hypothalamic–pituitary–adrenocortical (HPA) axis (Sapolsky, 1992). Besides controlling the HPA axis, the hypothalamus is also responsible for the integration of autonomic endocrine and even behavioral responses to stress (for a review, see Herman and Cullinan, 1997). Inputs from hypothalamic homeostasis get high priority, as the HPA system contributes to redistribution of bodily resources in times of physiologic need. HPA responses are affected by the cortex function. Restraint, fear conditioning or exposure to a novel environment, for example, are affected by lesions of the prefrontal cortex (for a review, see Herman and Cullinan, 1997). Exposure to a wide variety of stressors causes marked increase in neuronal and genomic activation of cortical neurons (for a review, see Sullivan and Gratton, 2002).

As mentioned above, extracellular adenosine concentrations have been observed to be increased upon stressful challenges (Latini and Pedata, 2001), including exposure to inescapable shock (Minor et al., 2001). Alterations of enzyme activities involved in nucleotide hydrolysis have also been reported in the spinal cord after repeated restraint stress (Torres et al., 2002). In this study, we investigated the effect of chronic restraint stress on the ATP, ADP and AMP hydrolysis in the blood serum, as well as ATP, ADP hydrolysis in synaptosomal fractions from the hypothalamus and cerebral cortex (two structures involved in the regulation of the HPA axis) of adult male Wistar rats.

2. Method

2.1. Animals

Adult male Wistar rats (60 days at the beginning of the treatment), weighing 150–230 g, were used. Experimentally naive animals were housed in groups of five in home cages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (light on between 07:00 and 19:00 h) at a room temperature of 22 ± 2 °C. The rats had free access to food (standard lab rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was performed between 10:00 and 12:00 h.

2.2. Chronic-restraint stress procedure

The animals were divided in two groups: stressed and control. Restraint was applied by placing the animals in a 25 × 7-cm plastic bottle and fixing it with plaster tape on the outside, so that the animal was unable to move. There was a 1-cm hole at one far end for breathing. The animals were stressed 1 h/day, 5 days/week for 45 days (Ely et al., 1997). After the stress procedure, the animals were returned to the home cages. Control animals were kept in their home cages during the period of treatment.

2.3. Subcellular fractionation

The animals were killed by decapitation 24 h after the last stress session. The brain was rapidly removed and the hypothalamus and cerebral cortex were dissected and gently homogenized in 10 vol of ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH = 7.5, with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously (Nagy et al., 1984). Briefly, 0.5 ml of the crude mitochondria fraction was mixed with 4.0 ml 8.5% Percoll solution and was layered onto an isosmotic Percoll sucrose discontinuous gradient (10%/20%). The synaptosomes that banded at the 10%/20% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The material was prepared fresh daily and maintained at 0–4 °C throughout the preparation.

2.4. Determination of ATP and ADP hydrolysis in synaptosomes from distinct brain structures

For brain structures, the reaction medium used to assay ATP and ADP hydrolysis was described previously (Battastini et al., 1991) and contained 5.0 mM KCl, 1.5 mM CaCl2, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The enzyme preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. For the evaluation of synaptosomes from the hypothalamus, a pool of hypothalamus from three rats was used in each assay. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min, and 100-μl samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986). Enzyme activities were expressed as nanomoles of phosphate released per minute per milligram of protein.

2.5. Isolation of the blood serum fraction

Trunk blood was drawn after decapitation of the animals, 24 h after the last stress session. Blood samples were centrifuged in plastic tubes for 5 min at 3000 × *g* at room temperature. The serum was separated, and it was used in the enzyme assay immediately.

2.6. Determination of ATP, ADP and AMP hydrolysis in the blood serum

ATP and ADP hydrolysis was performed using a modification of the method described by Yegutkin (1997). The reaction mixture containing ADP or ATP as substrate, 112.5 mM Tris–HCl, pH 8.0, was incubated with 1.0–1.5 mg protein serum at 37 °C in a final volume of 200 μl. The reaction was stopped by the addition of 200 μl 10% TCA. The amount of Pi liberated was measured by the method of Chan et al. (1986).

The reaction mixture containing AMP as a substrate in 100 mM Tris–HCl, pH 7.5, was incubated with 1.0–1.5 mg protein serum at 37 °C in a final volume of 200 μl. All other procedures were the same as for ATP and ADP hydrolysis, as described above.

For all enzyme assays, incubation times and protein concentration were chosen in pilot studies to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after the reaction was stopped with trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in duplicate. Enzyme activities were expressed as nanomoles of phosphate released per minute per milligram of protein.

2.7. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard.

2.8. Statistical analysis

Data were expressed by means ± standard error of the mean and analyzed by Student's *t* test.

3. Results

3.1. Experiment 1. Effect of chronic stress on hydrolysis of ATP and ADP in brain structures of rats

The effects of chronic stress on hydrolysis of ATP and ADP were assessed in brain structures 24 h after the last stress session. The hydrolysis of these nucleotides was not affected by chronic restraint stress in synaptosomes of hypothalamus and cerebral cortex (Student's *t* test, $P > .05$) (Table 1). The ATPase/ADPase ratio also showed no differences between groups (Student's *t* test, $P > .05$) (Table 1).

3.2. Experiment 2. Effect of chronic stress on hydrolysis of adenine nucleotides in the blood serum of rats

The effects of chronic stress on hydrolysis of ATP, ADP and AMP were evaluated in the blood serum. As shown in Table 2, ATPase and 5′-nucleotidase activities remained unaltered after chronic stress (Student's *t* test, $P > .05$). ADPase activity, however, was significantly decreased after

Table 1
Effect of chronic stress on ATP and ADP hydrolysis in synaptosomes from hypothalamus and cerebral cortex

	Hypothalamus		Cerebral cortex	
	Control	Stressed	Control	Stressed
ATPase	156.65 ± 20.56 (3)	166.06 ± 36.37 (3)	122.36 ± 9.89 (7)	121.99 ± 10.78 (7)
ADPase	27.41 ± 2.41 (3)	27.65 ± 1.78 (3)	23.47 ± 1.99 (7)	27.41 ± 2.41 (7)
ATPase/ADPase ratio	5.87 ± 0.25 (3)	5.88 ± 0.97 (3)	5.32 ± 0.40 (7)	4.14 ± 0.54 (7)

Values are means ± S.E.M. specific activity (picomoles of phosphate released per minute per milligram of protein). The number of assays per group is enclosed in parentheses. There were no differences between the groups (Student's *t* test, $P > .05$).

Table 2
Effect of chronic stress on the hydrolysis of ATP (ATPase), ADP (ADPase) and AMP (5′-nucleotidase), and on ATPase/ADPase ratio in the blood serum of rats

	Control	Stressed
ATPase	1.55 ± 0.17 (12)	1.47 ± 0.17 (11)
ADPase	1.76 ± 0.17 (12)	1.28 ± 0.12 (11) *
5′-Nucleotidase	1.73 ± 0.11 (17)	1.73 ± 0.06 (17)
ATPase/ADPase ratio	0.88 ± 0.04 (12)	1.15 ± 0.11 (11) **

The number of assays per group is enclosed in parentheses. Values are means ± S.E.M. specific activity (nanomoles of phosphate released per minute per milligram of protein).

* Significantly different from the control group (Student's t test, $P < .05$).

** Indicates significant difference from the ratio in the control group (Student's t test, $P < .05$).

chronic stress, remaining at 70% of control (Student's t test, $P < 0.01$). Accordingly, when these results are expressed as ATPase/ADPase ratios, it was observed an increased ratio in the blood serum of chronically stressed rats, consistent with the decreased ADPase activity (Table 2).

4. Discussion

In the present study, among the parameters studied, only the ADP hydrolysis in the blood serum was altered by chronic stress. When analyzing enzyme activities in the synaptosomal fraction of brain regions thought to be involved in the stress response, no effects were observed in chronically stressed rats, either on ATP or ADP hydrolysis. This was a surprising result, because adenosine is one of the main neuromodulators associated with cell stress (Cunha, 2001). Indeed, it is known that adenosine A1 receptors are induced by glucocorticoids (Svenningsson and Fredholm, 1997). Furthermore, the expression of mRNA for adenosine A1 receptors is significantly decreased after adrenalectomy (Svenningsson and Fredholm, 1997). Enhanced release of adenosine has also been demonstrated in brain tissue after exposure to stress. For example, inescapable shocks, as well as metabolic stress (glucoprivation by 2-deoxy-D-glucose administration), have been suggested to alter extracellular adenosine levels in the brain (Minor et al., 2001). In our work, no effect was observed in brain structures in the enzymes analyzed. Two points should be considered—that different stressors were used and, especially, that repeated restraint could lead to a process of adaptation that may cause different effects compared to those observed with acute stress. Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single stress episode do (Hashiguchi et al., 1997; Torres et al., 2001), and this phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes. A previous work showed that chronically stressed male rats present a decreased ADP hydrolysis in synaptosomes from the spinal cord (Torres et al., 2002). Therefore, alterations that can be observed in particular tissues or structures may reflect the different functions of the nucleotides and enzymes in different regions.

It was also shown that hydrolysis of ADP was altered in the blood serum of chronically stressed male rats. It has been demonstrated that stimulation of endothelial cells from umbilical vein by shear stress induces the release of endogenous adenosine triphosphate (ATP) (Bodin and Burnstock, 2001). It was suggested that the release of ATP from these cells, like that of nerve cells, is probably by vesicular exocytosis (Bodin and Burnstock, 2001). This phenomenon is accompanied by an extracellular increase in the activity of enzymes degrading both ATP (ATPases) and AMP (5′-nucleotidase) (Yegutkin and Burnstock, 2000). In addition, it was suggested that the enzymes, which are released during shear stress, are not released from an intracellular compartment together with ATP, but have an extracellular origin. In the present work, different results were observed, which could be determined by differences between in vitro and in vivo studies and between chronic and acute stress exposure.

In this study, it is important to note that the apparent dissociation between the two substrates (ATP and ADP) by the serum enzymes may be due to the simultaneous presence of two different enzymes involved in ATP hydrolysis, as described in other tissues, named an E-NTPDase and an ecto-ATPase (Zimmermann, 1996). However, just one of these two enzymes, the E-NTPDase, is implicated in ATP–ADP hydrolysis until AMP (Zimmermann, 1996). Inhibition of extracellular ADP hydrolysis, as observed in chronically stressed male rats, could induce decrease in adenosine production from extracellular ATP breakdown, because in this condition, there is a decrease in AMP levels and AMP is a substrate for 5′-nucleotidase.

Since we observed a difference in the activity of E-NTPDase in the serum of chronic stressed animals, and because there are different forms for this group of enzymes, probably, we are dealing with its soluble form, E-NTPDase 5 and/or E-NTPDase-6 (for a review, see Zimmermann, 2001). E-NTPDase-5 (or CD39-L4), for example, is secreted from mammalian cells and is soluble once secreted. It has specificity for NDPs over NTPs as substrates (Mulero et al., 1999). Its presence in macrophages indicates that this enzyme might be present in the blood and might have a role in modulating the levels of circulating ADP (Mulero et al., 1999). In addition, another nucleotide hydrolyzing enzyme has been reported to be present in the serum, the 5′-nucleotide phosphodiesterase (PDEase), which is able to promote the hydrolysis of both nucleotides, ADP and ATP (Sakura et al., 1998). The physiological function of this enzyme in the serum is still unclear, but it has been used as a marker of hepatoma (Tsou et al., 1982). The possible involvement of this enzyme in the hydrolysis of ADP cannot be completely discarded in our experimental conditions.

Considering time course determination for the inhibition of ADP hydrolysis, we have investigated the effect of acute stress on this parameter (to be published). No significant difference in ADP hydrolysis was observed immediately after 1 h restraint or 24 afterwards. Therefore, it is the repetition of the stress experience (chronic stress) that causes the decreases in ADP hydrolysis in the blood serum of rats at 24 h after the end of chronic stress treatment. It is not known, however, at what point of the 45 days of stress regimen this effect begins to manifest itself.

The ATPase/ADPase ratio may have an important role on the signaling properties of ATP (Chen and Guidotti, 2001). When an E-NTPDase is active, extracellular ATP is converted to AMP and then to adenosine by the action of a 5′-nucleotidase, and ADP is not an appreciable product. However, when E-NTPDase activity is reduced, as is the case in the blood serum from chronically stressed males, ATP could be converted to ADP by other ATPases, and this ADP would be relatively stable. Conversely, the ATPase activity did not change, probably due to an up-regulation of an ATPase that is coexpressed with the ATP diphosphohydrolase (Zimmermann, 1996; Sakura et al., 1998).

A cascade of nucleotidases may play a role in the effective regulation of several processes and may also have a protective function by keeping extracellular ATP and adenosine values within physiological levels (Agteresch et al., 1999). In the blood serum, adenine nucleotides have been implicated in several functions. ATP has been suggested to play a role in vascular tone, cardiac function and renal epithelial transport (Ravelic, 2000). In addition, other functions of extracellular adenine nucleotides include ADP-induced platelet aggregation (Hoylaerts et al., 2000). ADP is a potent platelet-recruiting factor and induces platelet aggregation via interaction with two P2 platelet receptors; a $P2Y_1$ receptor linked to phospholipase C pathways. ADP induces not only platelet shape change, exposure of fibrinogen binding sites and aggregation, but also the influx and intracellular mobilization of Ca^{2+}. The $P2Y_T/P2Y_{12}$ receptor is negatively coupled to adenylate cyclase, which mediates degranulation and sustained aggregation (for a review, see Puri, 1999). Hydrolysis of ADP by nucleotidases present in the serum inhibits platelet aggregation by removing ADP and by forming adenosine, which also inhibits aggregation (Zimmermann, 1999). In this context, the change of the ADP hydrolysis observed in the present study in the blood serum is very interesting. Since stress is one of the factors involved in atherosclerosis, and being ADP a signaling molecule, which activates platelet aggregation, the increase in ADP concentration in the serum of chronically stressed animals, as suggested here, may indicate the role of this factor in the etiology of atherosclerosis.

In conclusion, ADPase activity in the blood serum was altered in chronically stressed male rats. It is tempting to propose that the altered levels of ADPase activity in the serum may be a biochemical marker for chronic stress situations. Resolution of the possibility that it could be also a promoter of the stress-induced atherosclerosis will require additional work to further comprehend the involvement of nucleotide hydrolyzing enzymes in the blood of chronically stressed male rats.

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CAPÍTULO V

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The effect of stress upon hydrolysis adenine nucleotides in blood serum of rats

Ana Elisa Böhmer[a,1], Cristina Ribas Fürstenau[a,1], Iraci Lucena S. Torres[a], Leonardo Crema[a], Ana Maria O. Battastini[a], Carla Dalmaz[a], Maria Beatriz C. Ferreira[b], João J.F. Sarkis[a,*]

[a]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600 Anexo, Lab. 22, 90035-003 Porto Alegre, RS, Brazil*
[b]*Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, UFRGS, 90035-003 Porto Alegre, RS, Brazil*

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Abstract

Alterations of enzyme activities involved in adenine nucleotide hydrolysis have been reported in spinal cord and blood serum after repeated restraint stress. On the other hand, no effect was observed in the spinal cord of rats after acute stress. In the present study, we investigated the effect of acute stress on the hydrolysis of adenine nucleotides in rat blood serum. Adult male Wistar rats were submitted to 1-h restraint stress and were sacrificed at 0, 6, 24 and 48 h. Increased ATP and ADP hydrolysis were observed in the blood serum of stressed rats 24 h after stress (58% and 54%, respectively, when compared to controls). On the other hand, the AMP hydrolysis was increased after 6 h (68% when compared to controls) and at 24 h (94% when compared to controls) after stress. The results suggest that altered activity of soluble enzymes in serum may be a biochemical marker for stress situations.

Keywords: Apyrase; 5′-Nucleotidase; Stress; Nucleotide hydrolysis; Rat; Blood serum

1. Introduction

Extracellular ATP and its breakdown products, ADP and adenosine, have been shown to present pronounced effects on a variety of biological and pathological processes (Agteresch et al., 1999; Latini and Pedata, 2001). These effects of nucleotides were initially recognized in smooth muscle contraction, neurotransmission and regulation of cardiac function and platelet aggregation. Adenosine is particularly well suited to be used as a transcellular messenger to signal metabolic imbalance. Several reports have documented an increase in the extracellular concentration of adenosine upon stressful metabolic challenges (Latini and Pedata, 2001), and it has been suggested to have neuroprotective actions (for a review on adenosine, see Cunha, 2001).

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the surface, or may be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). Soluble nucleotidases, which can breakdown ATP and other adenosine nucleotides, have also been shown to be released from sympathetic nerves (Todorov et al., 1997). Works over the past few years have demonstrated that members of several families of ectonucleotidases can contribute to the extracellular hydrolysis of nucleotides. Nucleoside 5′-triphosphates and -diphosphates (NTP and NDP) may be hydrolyzed by members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP) family and by alkaline phosphatases (Zimmermann, 2001). These ectonucleotidases, together with 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this cascade formed by ectonucleotidases and 5′-nucleotidase is an enzymatic pathway with a double function of removing a signal of ATP and generating a

* Corresponding author. Tel.: +55-51-3316-5577; fax: +55-51-3316-5540.
E-mail address: jjsarkis@plug-in.com.br (J.J.F. Sarkis).
[1] Both are first authors.

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second one, adenosine. These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological conditions (Agteresch et al., 1999).

The physiological response to emotional or physical stress consists of an integration of endocrine and autonomic changes. In this situation, adrenomedullary epinephrine is released and hormones such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids (GCs) are released by the hypothalamic–pituitary–adrenocortical (HPA) axis (Sapolsky, 1992). The acute secretion of GCs is critical for responding to stress. Adenosine is one of the main neuromodulators associated with cell stress (Cunha, 2001). Indeed, it is known that adenosine A1 receptors are increased by GC (Svenningsson and Fredholm, 1997). The extracellular adenosine concentrations could be increased in stressful challenges (Latini and Pedata, 2001), including exposure to inescapable shock (Minor et al., 2001). Mild stress, such as a mild foot shock, is enough to promote specific changes in the ATP and ADP hydrolysis in some tissues such as the cerebral cortex (Pereira et al., 2002). Alterations of enzyme activities involved in nucleotide hydrolysis have also been reported in spinal cord and blood serum after repeated restraint stress (Torres et al., 2002a,b). In these studies, no effect was observed in the spinal cord of rats after acute stress. On the other hand, since increased ATP release has been reported after shear stress (Bodin and Burnstock, 2001), it is possible that the effects of repeated stress on the ATP hydrolysis cascade may be a consequence of an adaptation induced by the repetition of exposure to the stressor agent. In this sense, it would be important to evaluate the effects of acute stress on these processes. Therefore, in this study, we investigated the effect of restraint stress on ATP, ADP and AMP hydrolysis in the blood serum of adult male Wistar rats in different times after stress (0, 6, 24 and 48 h).

2. Methods

2.1. Animals

The study was performed in accordance with the University Ethics Committee guidelines for experiments with animals. Adult male Wistar rats (60 days at the beginning of the treatment), weighing 150–230 g, were used. Experimentally naive animals were housed in groups of five in home cages made of Plexiglas material ($65 \times 25 \times 15$ cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at a room temperature of 22 ± 2 °C. The rats had free access to food (standard laboratory rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was always performed between 10:00 and 12:00 a.m.

2.2. Stress procedure

The animals were divided into two groups: stressed and control. Restraint was applied by placing the animals in a 25×7-cm plastic bottle and fixing it with plaster tape on the outside so that the animals were unable to move. There was a 1-cm hole at one far end for breathing (Ely et al., 1997). The animals were sacrificed immediately, 6, 24 and 48 h after 1-h stress session. Control animals were kept in their home cage.

2.3. Isolation of blood serum fraction

Trunk blood was drawn by decapitation of the animals at 0, 6, 24 and 48 h after stress session. Blood samples were centrifuged in plastic tubes for 5 min at $3000 \times g$ at room temperature. Serum was separated and used in the enzyme assay immediately.

2.4. Enzyme assays

ATP and ADP hydrolyses were performed using a modification of the method described by Yegutkin (1997). The reaction mixture containing 0.5–1.0 mg protein serum in 112.5 mM Tris–HCl, pH 8.0, was preincubated during 10 min. ADP or ATP was used as substrate, and the incubation was performed at 37 °C in a final volume of 200 μl during 40 min. The reaction was stopped by the addition of 200 μl 10% trichloroacetic acid (TCA). The amount of P_i liberated was measured by the method of Chan et al. (1986).

The reaction mixture containing AMP as substrate in 100 mM Tris–HCl, pH 7.5, was incubated with 0.5–1.0 mg protein serum at 37 °C in a final volume of 200 ml. All other procedures were the same as described above for ATP and ADP hydrolysis.

For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after addition of TCA were used to correct nonenzymatic hydrolysis of the substrates. Enzyme activities were expressed as nanomoles of phosphate released per minute per milligram of protein.

2.5. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard.

2.6. Statistical analysis

Data were expressed as mean ± S.E.M. Groups were compared using Student's *t* test. Differences between experi-

mental and control groups were considered significant for $P < .05$.

3. Results

3.1. Effect of stress on ATP-ADPase activities in blood serum of rats

The hydrolysis of ATP and ADP were evaluated in the blood serum at 0, 6, 12 and 48 h after the stress procedure. When stressed animals were compared to the control group, ATPase and ADPase activities were significantly increased 24 h (58% and 54%, respectively, when compared with controls) after exposure to stress (Fig. 1; Student's t test, $P < .001$, and Student's t test, $P < .05$, respectively).

3.2. Effect of stress on 5′-nucleotidase activity in blood serum of rats

The hydrolysis of AMP was evaluated in the blood serum at 0, 6, 24 and 48 h after stress procedure. As shown in Fig. 2, 5′-nucleotidase activity was increased at 6 h (68%, Student's t test, $P < .02$) and 24 h (95%, Student's t test, $P < .005$) in the stressed group, always when compared to the control group.

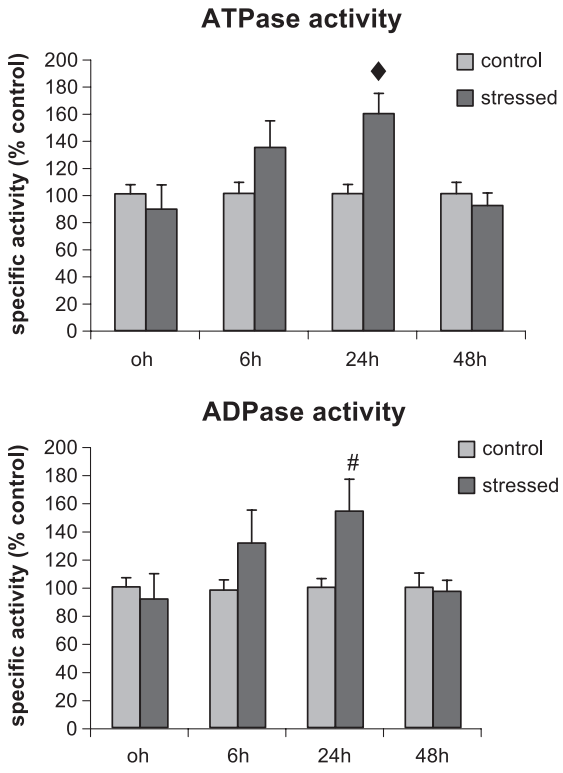

Fig. 1. Effect of acute stress on ATPase and ADPase activities in blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of phosphate produced/mg protein picomoles), considering the values of controls as 100% (absolute value for control groups: 0.92 ± 0.06 for ATPase and 0.99 ± 0.06 for ADPase activities). Number of animals per group = 6–11. #, significantly different from control group (Student's t test, $P < .05$). ◆, significantly different from control group (Student's t test, $P < .001$).

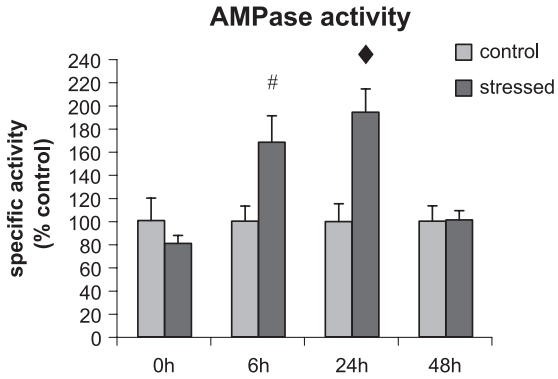

Fig. 2. Effect of acute stress on the 5′-nucleotidase activity in blood serum. Values are mean ± S.E.M. specific activity (nmoles of phosphate produced/mg protein), considering the values of controls as 100% (absolute value for control group: 0.05 ± 0.11). Number of animals per group = 5–8. #, significantly different from control group (Student's t test, $P < .02$). ◆, significant difference from the ratio in control group (Student's t test, $P < .005$).

4. Discussion

In the present study, ATPase, ADPase and 5′-nucleotidase activities in the blood serum were increased by acute restraint stress. A previous work showed that repeatedly stressed male rats present a decreased ADP hydrolysis in synaptosomes from the spinal cord (Torres et al., 2002a), with no effect when acute stress was utilized. Additionally, we also showed that only hydrolysis of ADP was altered in the blood serum of repeatedly stressed male rats (Torres et al., 2002b). Two points should be considered—that different models were used and especially that repeated restraint could lead to a process of adaptation that may cause different effects compared to those observed after acute stress. Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single stress episode do (Hashiguchi et al., 1997; Torres et al., 2001), and this phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes.

Nucleotidases have been shown to act in blood serum (Kaczmarek et al., 1996; Torres et al., 2002b). NTPDase 1 is associated with the surface of endothelial cells and smooth muscles, being located in their luminal surface (for a review, see Zimmermann, 2001). The enzymes whose activity was observed to be altered by restraint stress in the present study could be the soluble ones and/or those released from membranes, since they were measured in serum in vitro. However, considering the altered hydrolysis of nucleotides in serum after stress, when both ATP and ADP hydrolysis were increased with no change of ATPase/ADPase ratio, it is possible to consider that another soluble enzyme is involved in these alterations but not the E-NTPDase 5 and E-

NTPDase-6 because these enzymes have high preference for NDPs over NTPs as substrates (Mulero et al., 1999; Zimmermann, 2001). Confirming our proposal, other studies have suggested that nucleotidases released during shear stress may have an extracellular origin (Yegutkin et al., 2000), such as the membrane of cells.

The present results reinforce the possibility of one action site for both substrates in the serum enzyme (Oses et al., submitted for publication). In addition, another nucleotide-hydrolyzing enzyme has been reported to be present in serum, the 5′-nucleotide phosphodiesterase (PDEase), which is capable of hydrolyzing both nucleotides, ATP and ADP (Sakura et al., 1998). In this work, we do not discard the possibility of the involvement of a phosphodiesterase because we did not evaluate this activity using a specific substrate for this enzyme.

Circulating nucleotides are known to be important signaling molecules, potentiating a variety of physiological responses (Brake and Julius, 1996). In blood serum, adenine nucleotides have been implicated in several functions. ATP has been suggested to play a role in vascular tone, cardiac function and renal epithelial transport (Ravelic, 2000), and adenosine has been used clinically as an antiarrhythmic agent or vasodilatator. In addition, another function of extracellular adenine nucleotides is ADP-induced platelet aggregation (Hoylaerts et al., 2000). ADP is a potent platelet-recruiting factor and induces platelet aggregation via interaction with two P2 platelet receptors. ADP induces not only platelet shape change, exposure of fibrinogen binding sites and aggregation but also the influx and intracellular mobilization of Ca^{2+}. Hydrolysis of ADP by nucleotidases present in the serum inhibits platelet aggregation by removing ADP and forming adenosine, which, besides other effects, inhibits platelet aggregation (Zimmermann, 1999). On the other hand, stress is known to trigger a hypercoagulable state, probably mediated by plasma catecholamine activity (Von Känel et al., 2002). In this sense, the response observed in the present study with serum after restraint stress may contribute to reduce these procoagulant effects, being a protective mechanism against acute coronary thrombosis and atherosclerosis development. Soluble CD39 (SolCD39), for example, improves cerebral blood flow and reduces cerebral infarct volume when given preoperatively (Pinsky et al., 2002). However, it is important to consider that repeated restraint lead to opposite effects, with decreased ADP hydrolysis in blood serum (Torres et al., 2002b). In this context, the alterations of ADP concentration in serum of stressed animals, showing increase with repeated stress and decrease with acute stress, may indicate the role of this factor in the etiology of cardiovascular diseases.

The ATPase/ADPase ratio may have an important role in the signaling properties of ATP (Chen and Guidotti, 2001). When an E-NTPDase is active, extracellular ATP is converted to AMP and then to adenosine by the action of a 5′-nucleotidase, and ADP is not an appreciable product. Because 5′-nucleotidase is also increased after restraint stress, the resulting effect is a decrease in one signal evoked by ATP and an increase in another signal evoked by adenosine. In addition, our results suggest that these enhanced adenosine levels may remain high in serum several hours after exposure to stress.

The reaction catalyzed by 5′-nucleotidase is the rate-limiting step in this extracellular pathway from ATP to adenosine (for a review, see Cunha and Ribeiro, 2000). It is important to observe that this enzyme is inhibited by ATP and/or ADP (Cunha and Sebastião, 1991). Therefore, only when ATP and ADP levels decrease below the threshold of inhibition of 5′-nucleotidase will an important amount of adenosine be formed. In this context, it is important to consider that in the present study, the hydrolysis of ATP and ADP were also increased after stress, giving the possibility to 5′-nucleotidase to act, producing adenosine.

Several biological and mechanical stressors may induce endogenous self-protective mechanisms to avoid cellular injury. For example, adenosine may act as an endogenous cardioprotective substance in pathophysiological conditions of the heart, such as ischemia (for a review, see Kitazake et al., 1999). Adenosine release during ischemia is beneficial by proving receptor-mediated vasodilatory protection and has also been shown to mediate ischemic preconditioning (Downey et al., 1993). Both ecto-5′-nucleotidase activity and adenosine levels are increased in blood and in myocardium in patients with chronic heart failure (for a review, see Kitazake et al., 1999). These reports suggest adenosine as a protective factor after stress situations.

In conclusion, ATPase, ADPase and 5′-nucleotidase activities in blood serum were altered in stressed male rats. These effects may represent a protective mechanism against some of the stress effects. It is tempting to propose that the altered ectonucleotidase activities in serum may be a biochemical marker for stress situations.

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DISCUSSÃO

Dor é fenômeno complexo que envolve vários sistemas de neurotransmissão e neuromodulação, sendo de fundamental importância que estudos sejam desenvolvidos para melhor entender esses processos.

Enquanto existem vários modelos animais de dor aguda, há poucos de dor crônica, envolvendo habitualmente destruição tecidual. O modelo proposto nessa tese - estresse crônico por imobilização - pode ser uma opção para estudos na área de dor crônica, pois, sem ser invasivo e sem causar maiores prejuizos ao animal, alterou o limiar nociceptivo, produzindo hiperalgesia (Gamaro *et al.*, 1998, Torres *et al.*, 2001a). E, além da hiperalgesia, os estudos farmacológicos realizados nessa tese somados a estudos anteriores do grupo sugerem que o estresse crônico leva a alterações em nível de dois sistemas muito importantes na modulação da dor, sistemas opióide e adenosinérgico.

Nos experimentos descritos no capítulo I, teve-se como objetivo determinar se o modelo de imobilização crônica pode alterar a analgesia induzida por estresse agudo (*stress-induced analgesia,* SIA). Utilizaram-se, para tanto, dois tipos de estressores - natação e imobilização. Observou-se que os animais cronicamente estressados não apresentaram a resposta analgésica característica, após serem submetidos a estresse agudo por imobilização. No entanto, essa resposta estava presente quando esses animais foram submetidos a estresse agudo por natação forçada.

Siegel (1977) sugeriu que animais testados em um novo contexto mostram respostas analgésicas, enquanto animais testados no contexto original continuam a exibir tolerância à analgesia. Resultados dessa tese corroboram esses dados, visto que, quando foi empregado o mesmo estressor do tratamento crônico, não se observou resposta analgésica nos animais estressados cronicamente, enquanto

animais do grupo controle apresentaram o efeito antinociceptivo clássico. Isso ocorreu provavelmente devido à adaptação dos animais estressados por imobilização a esse tipo de estressor. Por outro lado, estresse agudo por natação forçada produziu efeito analgésico em ambos os grupos. Esses resultados sugerem que animais cronicamente estressados são capazes de responder a estresse com resposta analgésica. No entanto, não respondem quando o estressor for o mesmo utilizado no tratamento crônico. Blustein e colaboradores (1995) sugerem que tolerância a formas de SIA mediadas contextualmente por repetida exposição a estímulos pode ser conseqüência de prolongada ativação opióide e subseqüente alteração desse sistema.

No modelo empregado nessa tese, observou-se que animais repetidamente estressados apresentavam diminuição dos efeitos de morfina sobre nocicepção, quando comparados a animais não-estressados. Aqueles necessitaram de dose mais alta e maior período de tempo para que fosse obtido o efeito analgésico clássico da morfina (Torres, 1999). Mudanças em sensibilidade para antinocicepção opióide observadas nesses ratos podem ser decorrentes de alterações em receptores opióides centrais ou periféricos, em número ou afinidade. Podem ainda resultar de alterações em outros sistemas hormonais ou de neurotransmissores capazes de interagir com receptores opióides. Estudo mostrou que esses animais podem apresentar diminuição em densidade de receptores opióides em sistema nervoso central (córtex cerebral, hipocampo e medula espinhal) (Dantas *et al.*, comunicação pessoal). Uma vez que a morfina exerce seus efeitos antinociceptivos primariamente por meio de receptores de subtipo μ, a resposta alterada observada em animais submetidos a estresse crônico pode ser resultante de mudanças nos níveis desses receptores. Persistente ativação dos receptores por opióides

endógenos liberados durante o estresse crônico por imobilização pode levar ao fenômeno de *down-regulation,* alterando a sensibilidade à morfina. Aquele fenômeno é observado com o uso crônico de analgésicos opiódes na clínica médica (Gutdtein & Akil, 2001). Propõe-se assim que nesse modelo haja uma menor atividade opióide.

Estudos sobre fenômeno de SIA têm demonstrado envolvimento de múltiplos sistemas inibitórios de dor (Lewis *et al.*, 1982, Bodnar & Sirorszky, 1983, Rochford & Stewart, 1987). Resposta analgésica ao estresse tem sido tipicamente caracterizada em termos de sua mediação por mecanismo opióides e não-opióides (Lewis *et al.*, 1980, Lewis *et al.*, 1982, Bodnar, 1986, Rochford & Stewart, 1987, Terman & Bonica, 2001). Isto é, distintos mecanismos neuroquímicos inibitórios de dor podem ser ativados, dependendo de propriedades do estressor, como por exemplo, intensidade e duração (Lewis *et al.*, 1980, Terman *et al.*, 1986, Mogil *et al.*, 1996), além de outros fatores (linhagem e gênero do animal) (Urca *et al.*, 1985, Gamaro *et al.*, 1998). Classicamente estresse induz resposta analgésica com participação opióide (Pohorecky *et al.*, 1999). No entanto, em estudos em que analgesia por estresse é induzida por meio de natação (SSIA - *swim stress-induced analgesia*) em camundongos e ratos, manipulação de parâmetros como temperatura da água e tempo de natação parece modificar a resposta a estresse e o papel de diferentes vias inibitórias de dor responsivas à SIA. Assim, podem ser vistas tipos de SIA opióides e não-opióides (Mogil *et al.*, 1996, Hopkins *et al.*, 1998, Omiya *et al.*, 2000). É possível que a analgesia produzida por nado forçado, observada nesse estudo, seja uma forma de SIA não-opióide. Dessa forma, não se observou analgesia por estresse agudo com estressor habitual, mas aquela ocorreu com estressor distinto, talvez não sendo mediado por sistema opióide.

A hiperalgesia observada nos animais cronicamente estressados é de longa duração, uma vez que não é revertida com a suspensão do tratamento crônico por 14 ou 28 dias. Este efeito pode ser resultante de tolerância de longa duração do eixo HPA, alteração em receptores opióides e/ou alteração em outros sistemas relacionados à resposta ao estresse, que envolvam algum tipo de plasticidade neural semelhante à observada em aprendizado e memória. Por outro lado, a resposta ao estresse agudo por imobilização foi restabelecida após 28 dias de suspensão do tratamento, embora os animais continuassem hiperalgésicos na medida basal. Portanto, o restabelecimento dos sistemas envolvidos na resposta nociceptiva tem diferentes padrões, variando de acordo com período de tempo e resposta avaliada (medida basal ou resposta nociceptiva ao estresse agudo).

Sugere-se que estresse prolongado pode levar a alterações duradouras no sistema neural envolvido com modulação nociceptiva, que persistem após suspensão do tratamento. Trabalhos em seres humanos mostram que redução nos limiares de dor após longo período de estresse psico-emocional pode se dever à redução da atividade de sistema opióide cerebral (Ashkinazi & Vershinina, 1999). Outros estudos também sugerem envolvimento de sistema opióide na resposta hiperalgésica induzida por estresse crônico por imobilização (Gamaro *et al*, 1998, Torres *et al.*, 2001a).

Resultados de experimentos suportam hipótese de que adenosina pode estar envolvida com efeitos farmacológicos da morfina (Cahill *et al.*, 1995, Capasso, 1999, Zarrindast *et al.*, 1999). Nos experimentos do capítulo II, demonstrou-se que animais cronicamente estressados não apresentam resposta analgésica após administração de CPA e dipiridamol, efeito que foi observado em animais controles. Adicionalmente, resposta antinociceptiva de CPA em controles foi revertida por

DPCPX, um antagonista de receptor A1 de adenosina, sugerindo que aquele efeito é mediado por esses receptores. Efeito antinociceptivo de dipiridamol foi também revertido por DPCPX, corroborando a hipótese de que há envolvimento da adenosina na resposta antinociceptiva observada em animais controles. Ausência de efeito em animais cronicamente estressados indica diferente modulação do processo nociceptivo induzida por estresse crônico e que esta modulação envolve sistema adenosinégico. Sugere também que alterações em receptores A1 de adenosina possam estar envolvidas na hiperalgesia observada em animais cronicamente estressados.

Estudos têm mostrado que camundongos sem receptor A1 de adenosina mostram respostas hiperalgésicas (Johansson *et al.*, 2001). Com base em antagonismo, tolerância e abstinência cruzada entre esses sistemas, receptor A1 de adenosina tem sido proposto como parte de um complexo multireceptor opióide μ e α_2-adrenérgico (Aley & Levine, 1997).

Morfina produz liberação de adenosina dependente de dose e mediada por receptor opióide em sinaptossomas de medula espinhal. Esta liberação ocorre por meio de transportadores de nucleosídeo sensíveis a dipiridamol, mas insensíveis a nitrobenziltioinisina (Sandner-Kiesling *et al.*, 2001). O processo de transporte de nucleosídeo pode ter papel na regulação dos níveis endógenos de adenosina em sistema nervoso central. Inibição de transportadores de nucleosídeo pode aumentar significativamente a concentração de adenosina extracelular, provavelmente devido à inibição da captação de adenosina resultante da hidrólise de nucleotídeos. A inibição da captação pode aumentar os níveis de adenosina na fenda sináptica e subseqüentemente aumentar a ativação de receptores extracelulares de adenosina, o que pode levar à resposta antinociceptiva (Sweeney *et al.*, 1993). Zarrindast e

colaboradores (1993) demonstraram que administração de inibidores de transportadores de nucleosídeos induz efeito antinociceptivo em ratos; no entanto, esse efeito não foi observado em animais estressados cronicamente. Uma vez que facilitação do sistema adenosinérgico pode induzir antinocicepção e sua inibição pode facilitar neurotransmissão nociceptiva, possivelmente a modulação adenosinérgica esteja alterada naqueles animais.

Análogos de adenosina têm propriedades antinociceptivas em situações clínicas, como em dores neuropáticas, e experimentais, em que mecanismos sinalizadores de dor estão alterados (Sawynok, 1998, Jarvis & Kowaluk, 2001). Guieu e colaboradores (1996) demostraram reduzidos nível de adenosina em sangue e líquor de pacientes com dor neuropática. Por outro lado, há relatos de ausência de efeito com administração intratecal de adenosina para alívio de comportamento semelhante à alodinia (von Heijne *et al.*, 1999).

Assim, os efeitos das purinas na nocicepção são complexos, dependendo do subtipo de receptor ativado e do sítio de administração dessas substâncias. Os receptores de adenosina de subtipo A1 estão associados a efeito modulatório na transmissão da dor em medula espinhal (Keil & DeLander, 1996). Agonistas A1 parecem atuar pré-sinapticamente, inibindo a liberação de neurotransmissores, ou pós-sinapticamente, reduzindo a excitabilidade neuronal (Hass & Greene, 1988, Lamber & Teyler, 1991, Poli *et al.*, 1991). Por outro lado, a atuação de adenosina em receptores A2 aumenta a liberação de neurotransmissores (Cunha *et al.*, 1992, Tsuda *et al.*, 1999). Medula espinhal dorsal contém tanto receptores de adenosina A1 quanto A2 (Goodman & Snyder, 1982, Choca et al., 1987, Sawynok & Sweeney, 1989).

Experimentos dessa tese sugerem que estresse crônico por imobilização interfere na resposta nociceptiva por alterações em sistemas opióides e adenosinérgico, que teriam sua atividade reduzida.

Animais cronicamente estressados, como demonstrado nos experimentos dos Capítulos III e IV, apresentaram redução da atividade ADPásica tanto em medula espinhal quanto em soro, e aumento da atividade 5'nucleotidásica em medula espinhal. Esses efeitos sugerem presença de menores níveis de adenosina nessas frações, e o aumento da atividade da 5'nucleotidase em medula espinhal poderia resultar de um efeito compensatório para a conseqüente diminuição dos níveis de AMP. Os animais agudamente estressados não apresentaram alteração nas atividades das enzimas analisadas em medula espinhal, mas houve aumento dessas atividades no soro.

Aparente dissociação entre os dois substratos (ATP e ADP) pode ser conseqüente à presença simultânea de duas diferentes enzimas envolvidas na hidrólise do ATP, chamadas de E-NTPDase e ecto-ATPase (Zimmermann, 1996), mas somente de uma delas, E-NTPDase, na hidrólise ATP-ADP até AMP (Zimmerman, 1996). Inibição da atividade ADPásica observada em animais cronicamente estressados poderia levar à diminuição na produção de adenosina resultante da quebra do ATP, uma vez que, nesta condição, há diminuíção dos níveis de AMP, que é substrato para 5'nucleotidase.

A presença de diferentes populações de ectonucleotidases é também suportada pelo aumento da razão ATPase/ADPase observada em sinaptossomas de medula espinhal e soro de animais cronicamente estressados. Quando E-NTPDase 1 (CD39, ecto-apirase, ecto-ATP difosfo-hidrolase) está ativa, converte ATP extracelular a ADP e, posteriormente, a AMP. Este, por sua vez, é convertido em

adenosina pela ação da 5'-nucleotidase, de modo que o ADP não é produzido em quantidades significativas. No entanto, quando a atividade de E-NTPDase 1 está reduzida, como é o caso de medula espinhal e soro de ratos cronicamente estressados, ATP pode ser convertido a ADP por outras ecto-ATPases, e este último pode ser relativamente estável. Conseqüentemente, a atividade ATPásica não muda, provavelmente devido ao fenômeno de *up-regulation* da ecto-ATPase, que é co-expressada com ecto-ATPdifosfo-hidrolase (Zimmermann, 1996, Sakura *et al.*, 1998).

Animais cronicamente estressados não apresentaram qualquer alteração nas frações sinaptossomais das regiões cerebrais estudadas, apesar de córtex cerebral e hipotálamo serem estruturas envolvidas com a resposta ao estresse (Capítulo IV). Este foi um resultado surpreendente, uma vez que adenosina é um dos principais neuromoduladores associados a estresse celular (Cunha, 2001). A modulação adenosinérgica por receptores A1/A2 está presente em diferentes regiões de SNC, e diferentes efeitos têm sido observados quando distintas regiões cerebrais são consideradas (Lamber & Teyler, 1991, Thompson *et al.*, 1993).

Sabe-se que receptores A1 de adenosina são induzidos por GCs (Svenningsson & Fredholm, 1997). Além disso, expressão do RNAm para esses receptores é significativamente aumentada após adrenalectomia (Svenningsson & Fredholm, 1997). Maior liberação de adenosina também tem sido demonstrada em tecido cerebral após exposição a estresse. Por exemplo, choque inescapável e estresse metabólico (privação de glicose pela administração de 2-deoxi-D-glicose) alteram níveis extracelulares de adenosina no cérebro (Minor *et al.*, 2001). Como no presente estudo nenhum efeito foi observado nas estruturas cerebrais analisadas, dois pontos podem ser considerados. Estressores utilizados nessa tese e nos

estudos prévios foram diferentes e, especialmente aqui, foi empregado estresse crônico que pode levar a processos adaptativos distintos daqueles produzidos por estressores agudos. Os animais cronicamente estressados não estão submetidos às mesmas repercussões hormonais que os animais expostos a um único episódio de estresse (Tizabi & Aguilera, 1992, Hashiguchi *et al.*, 1997, Torres *et al.*, 2001b), e esse fenômeno de adaptação ao estresse crônico pode se refletir em vários processos bioquímicos e fisiológicos.

Uma vez que animais cronicamente estressados apresentaram diminuição na atividade ADPásica em sinaptossomas de medula espinhal e soro, sem alteração em córtex e hipotálamo, sugere-se que as alterações observadas em determinadas frações e/ou estruturas podem refletir diferentes funções dos nucleotídeos e enzimas em diferentes regiões.

O papel de ATP em nocicepção foi proposto com base em experimentos em seres humanos, após a formulação da hipótese purinérgica. O receptor $P2X_3$ é de particular interesse no contexto de vias de dor, porque é seletivamente expresso em altos níveis por neurônios nociceptivos sensoriais (Ding *et al.*, 2000). Além do papel excitatório e em neurônios sensoriais, este receptor pode ter papel adicional pré-sinapticamente, regulando a liberação de glutamato de neurônios aferentes primários no corno dorsal da medula espinhal (Ding *et al.*, 2000).

Há evidências que ATP pode mediar dor isquêmica durante angina ou enxaqueca e menorragia em mulheres. Plaquetas são ricas em ATP, e a agregação plaquetária promove sua liberação. Agregação plaquetária ocorre em enxaqueca, e o ATP liberado pode contribuir para a dor de enxaqueca (Burnstock & Wood, 1996). Ferguson e colaboradores (1997) demonstraram também liberação dessa purina de células uroteliais na bexiga, como resultado de estimulação elétrica ou estímulo

estressor. Portanto, o ATP liberado de diferentes tipos celulares parece estar envolvido com inicialização de dor pela ação em puriceptores de teminações nervosas sensoriais (Burnstock, 1996).

Estudos em animais e seres humanos mostram que alguns estados de dor neuropática são "mantidos simpaticamente", isto é, são exacerbados pela atividade de neurônios simpáticos. Uma vez que receptores para ATP são encontrados em neurônios sensoriais, tanto perifericamente quanto em corno da raiz dorsal, é possível sugerir que ATP é mediador de respostas sensoriais simpáticas anormais (para revisão, ver Hamilton & Mcmahon, 2000).

Animais cronicamente estressados têm o eixo simpático-adrenal ativado. Portanto, pode haver importante papel do ATP na hiperalgesia apresentada por eles. Além disso, hidrólise de ADP *in vitro* está diminuída em medula espinhal, sugerindo inibição em atividade ADPásica *in vivo.* Isto pode estar promovendo aumento na concentração de ATP extracelular nesses animais e este ATP pode estar atuando como neuromodulador ou neurotransmissor excitatório em sinapses do corno dorsal. Por outro lado, aumento da concentração de ADP, resultante de diminuição de sua hidrólise em soro de ratos estressados cronicamente, pode estar promovendo agregação plaquetária. Isso leva à liberação de ATP, que pode atuar como ativador periférico de neurônios sensoriais nociceptivos por meio de receptores de ATP ligado a canais iônicos.

Sabe-se que o controle da atividade de tecidos endócrinos, incluindo medula adrenal, pâncreas endócrino e hipófise anterior, pode ser outra função extracelular exercida pelo ATP em situações de estresse. ATP é co-liberado com produtos secretórios de células cromafínicas de adrenal (Rojas *et al.*, 1985) e células β-pancreáticas (Furomi *et al.*, 1995). É possível considerar que os resultados dessa

tese, mostrando redução na atividade ADPásica, representem uma adaptação no funcionamento dessas glândulas. Nucleotidases podem também controlar a disponibilidade de ligantes (ATP, ADP, AMP) para receptores de nucleotídeos e conseqüentemente, duração e extensão da ativação do receptor (Chen & Guidotti, 2001).

Alteração na atividade de E-NTPDase observada em soro de ratos cronicamente estressados provavelmente reflete repercussões sobre formas solúveis das enzimas E-NTPDase 5 e/ou E-NTPDase-6. Estas são ativadas por Ca^{2+} ou Mg^{2+} e têm alta preferência por hidrolisar nucleotídeos difosfatados (para revisão, ver Zimmermann, 2001). E-NTPDase-5 (ou CD39-L4), por exemplo, é secretada de células de mamíferos e, uma vez secretada, é solúvel. Esta enzima tem especificidade para NDPs sobre NTPs como substratos (Mulero *et al.*, 1999). Sua presença em macrófagos indica possível ocorrência também em sangue. Tem papel na modulação dos níveis de ADP circulante (Mulero *et al.*, 1999). E-NTPDase-6 (ou CD39-L2) está associada a complexo de Golgi e, em menor grau, à membrana plamática. É, assim como a NTPDase-5, liberada em meio de cultura (para revisão ver Zimmermann, 2001). Além disto, outra enzima que hidrolisa nucleotídeo pode estar presente no soro, a 5'-nucleotideo fosfodiesterase (PDEase), que é capaz de hidrolisar tanto ADP quanto ATP (Sakura *et al.*, 1998). A função fisiológica desta enzima no soro não está clara, mas tem sido usada como marcador de hepatoma (Haugen *et al.*, 1981; Tsou *et al.*, 1982). O possível envolvimento desta enzima na hidrólise do ADP não pode ser completamente descartado nas condições experimentais aqui descritas.

A cascata de nucleotidases tem papel efetivo na regulação de vários processos, incluindo função protetora na manutenção de ATP (extracelular) e

adenosina dentro de níveis fisiológicos (Agteresch *et al.*, 1999). Nucleotídeos circulantes são importantes moléculas sinalizadoras, potencializando várias respostas fisiológicas (Brake & Julius, 1996). Em soro, os nucleotídeos de adenina estão implicados em vários processos. ATP tem função em tônus vascular, função cardíaca e transporte epitelial renal (Chen *et al.*, 1994, Inscho *et al.*, 1994, Nuñez *et al.*, 1995, Boarder *et al.*, 1995, Ravelic, 2000). Adenosina é usada clinicamente como agente antiarrítmico ou vasodilatador. ADP é potente fator de recrutamento de plaquetas e induz agregação plaquetária por meio da interação com dois tipos de receptores P2 plaquetários - $P2Y_1$ e P2T ($P2Y_T/P2Y_{12}$) (Burnstock & Williams, 2000). Hidrólise de ADP por ecto-nucleotidases presentes em soro inibe agregação plaquetária por remoção de ADP e formação de adenosina (também inibidora da agregação plaquetária) (Zimmerman, 1999). CD39-L4 solúvel no sangue melhora fluxo cerebral e reduz volume de infarto cerebral, quando administrada pré-operatoriamente (Pinsky *et al.*, 2002).

Sabe-se que respostas cardiovasculares exageradas a estresse e freqüente exposição a episódios estressantes podem ser fator de risco e/ou marcador de doença cardiovascular (Light *et al.* 1999). O estresse como indutor de ativação de sistemas envolvidos em hemostase pode estar envolvido no desencadeamento de sindromes coronárias agudas. Modelos de estresse em laboratório mostram aumento de pressão arterial (Matthews *et al*, 1993, Zhu *et al*, 2002) e prejuízo de fluxo sangüíneo (Lind *et al.*, 2002). Concentrações de ADP em soro de animais estressados, que se mostraram aumentadas em estresse crônico e diminuídas em estresse agudo, podem ser importantes indicativos para papel de estresse na etiologia de doenças cardiovasculares. Uma vez que estresse crônico é fator envolvido em aterosclerose e sendo ADP uma molécula sinalizadora que ativa

agregação plaquetária, o aumento da concentração de ADP em conseqüência da diminuição de sua hidrólise pode indicar um papel deste fator na etiologia da aterosclerose.

No capítulo V, observou-se que a atividade de 5'-nucleotidase está aumentada após estresse agudo, o que poderia resultar em diminuição do sinal evocado pelo ATP e aumento do sinal evocado pela adenosina. Aquele aumento persistiu por várias horas após a exposição ao estresse, sendo ainda observado em 6 h, enquanto a alteração das atividades ATPásica e ADPásica foi observada somente 24 h após o estresse.

A reação catalisada por 5'nucleotidase é passo limitante nas vias extracelulares que levam à formação de adenosina a partir de ATP (Cunha & Ribeiro, 2000). Esta enzima é inibida por ATP e/ou ADP (Cunha & Sebastião, 1991). Assim, somente quando níveis de ATP e ADP diminuem, será formada quantidade importante de adenosina. Em estresse agudo, a hidrólise de ATP e ADP também estão aumentadas, possibilitando a atuação de 5'nucleotidase e a produção de adenosina.

Alteração observada na hidrólise do AMP pode resultar em aumento na concentração sérica de adenosina. Este pode ser um dos mecanismos envolvido na analgesia observada após estresse agudo, uma vez que se sabe que essa analgesia pode ser de natureza opióide ou não-opióide. Este efeito é contrário ao que se observou em estresse crônico, em que houve diminuição da hidrólise do ADP, o que promoverá menores níveis de AMP (substrato para a 5'nucleotidase) e, possivelmente, diminuição dos níveis séricos de adenosina. Isto favoreceria a resposta hiperalgésica observada.

Além de repercussões em resposta nociceptiva, exposição a estressores pode também levar a alterações em fenômenos vasculares. Estresse agudo pode contribuir para reduzir efeitos pró-coagulantes do ADP, sendo um mecanismo protetor contra trombose coronariana e desenvolvimento de aterosclerose, uma vez que induz aumento na hidrólise de todos os nucleotídeos. Vários estressores biológicos e mecânicos podem induzir mecanismos de auto-proteção para evitar injúria celular. Por exemplo, a adenosina pode atuar como substância cardioprotetora em condições como isquemia (para revisão, ver Kitakaze *et al.*, 1999). Adenosina liberada durante isquemia produz proteção vasodilatadora por meio de ação em seus receptores específicos. Tanto a atividade da 5'nucleotidase quanto os níveis de adenosina estão aumentados em sangue e miocárdio, em pacientes com insuficiência cardíaca crônica (para revisão, ver Kitakaze *et al.*, 1999). Esses dados sugerem que a adenosina atue como um fator protetor após situações de estresse agudo.

CONCLUINDO a hiperalgesia induzida por estresse crônico por imobilização em ratos persistiu por pelo menos 28 dias após a suspensão do tratamento. Quando os animais foram submetidos a uma nova sessão de estresse agudo ao final do tratamento crônico, antinocicepção foi observada somente após exposição a diferente agente estressor (natação forçada). A clássica resposta analgésica ao estresse foi restabelecida após 28 dias de interrupção do tratamento, embora os animais continuassem apresentando diminuição no limiar nociceptivo. Foi demonstrado que animais cronicamente estressados desenvolveramtolerância a morfina e análogos de adenosina. Eles apresentaram também diminuição na hidrólise do ADP em medula espinhal e soro, o que possivelmente levou à

diminuição dos níveis de adenosina. Isto poderia estar relacionado aos efeitos comportamentais e farmacológicos observados.

Redução de atividade ADPásica sérica e medular pode ser um marcador bioquímico para situações de estresse crônico. Sistemicamente, o estresse pode ser um promotor de aterosclerose devido ao aumento dos níveis de ADP circulante, sabidamente indutor de agregação plaquetária. Futuros trabalhos são necessários para melhor determinar essas relações.

Na figura 4, é apresentado um esquema geral que procura sistematizar os dados obtidos nesse trabalho de tese em relação à nocicepção dos animais cronicamente estressados, incorporando-os aos previamente obtidos. Estabeleceu-se assim a hipótese de que o estresse crônico por imobilização leva à redução da atividade do complexo receptor opióide-adenosinérgico-adrenérgico. A dessensibilização opióide e adenosinérgica justificaria vários achados comportamentais e farmacológicos. Como etapa seguinte, seria interessante estudar o papel do sistema adrenérgico nesse modelo.

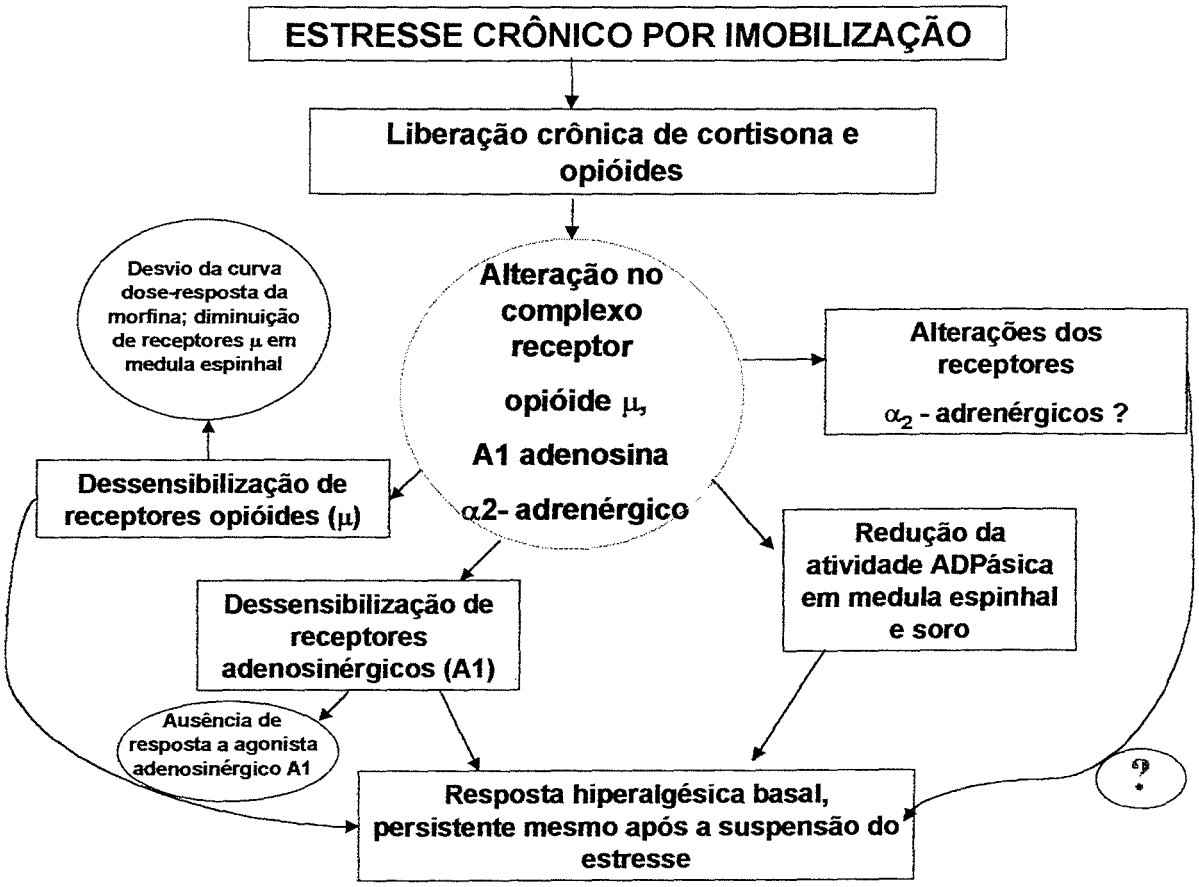

Figura 4: Esquema geral representativo da hipótese de alteração do complexo receptor opióide μ, adenosina A1e α_2-adrenérgico no modelo de estresse crônico por imobilização.

CONCLUSÃO

Os resultados dessa tese levam às conclusões a seguir:

- Estresse crônico por imobilização induziu hiperalgesia e alterou a resposta analgésica clássica a estresse agudo homotípico.

- A hiperalgesia observada após estresse crônico por imobilização pode ser resultado de alterações no sistema adenosinérgico, mais especificamente em receptor A_1.

- Animais machos cronicamente estressados por imobilização apresentaram diminuição na atividade ADPásica em medula espinhal e este efeito pode estar envolvido com a hiperalgesia por eles apresentada. Paralelamente, tal alteração não foi observada em fêmeas estressadas cronicamente, e estas não apresentaram hiperalgesia.

- Estresse crônico por imobilização não induziu alterações de atividade ATPásica/ADPásica em córtex cerebral ou hipotálamo.

- Animais cronicamente estressados por imobilização apresentaram diminuição da atividade ADPásica em soro, e este efeito pode estar envolvido com a resposta hiperalgésica observada e com o fato do estresse ser um possível promotor de aterosclerose.

- Estresse agudo por imobilização não induziu alterações de atividade ATPásica/ADPásica em medula espinhal de machos ou fêmeas.

- Estresse agudo por imobilização induziu aumento das atividades ATPásica/ADPásica e de 5'nucleotidase em soro de ratos, efeito este que pode estar envolvido com a analgesia induzida por estresse agudo e com processos protetores cardiovasculares, resultantes do aumento da adenosina circulante.

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ANEXO

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