Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós-Graduação de Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Avaliação dos efeitos da homocisteína em tecidos cardíaco e cerebral (*ex vivo*) e em cultura de astrócitos adultos – possível papel protetor da vitamina D

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Orientadora: Profa Dra Angela Terezinha de Souza Wyse

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Porto Alegre, 2016.

"Um cientista em um laboratório não é um mero técnico: ele também é uma criança que confronta fenômenos naturais que o impressionam como se fossem contos de fada."

[Marie Curie – 1867 - 1934]

Dedico essa tese às pessoas mais importantes da minha vida, meu pai, Dario, e minha mãe, Ivete, por ser meu porto seguro sempre, pela doação, incentivo e força.
Meu marido, Adriano.
E ao grande amor, meu filho, Bernardo.

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A TODOS, obrigada de coração.

SUMÁRIO

RESUMO

As hiperhomocisteinemias (HHcy), leve e moderada são consideradas um fator de risco para doenças cardiovasculares e cerebrais, entretanto os mecanismos e as complicações decorrentes dessa condição ainda não estão bem estabelecidos. Ela ocorre em 5-10% da população geral e em 40% dos pacientes com doenças vascular periférica e doenças cerebrovasculares. Estudos recentes têm demonstrado que a vitamina D (calcitriol) possui efeitos protetores em diversos modelos experimentais que enfatizam suas possíveis ações antioxidantes. O objetivo principal dessa tese de doutorado foi estabelecer um protocolo experimental com diferentes concentrações de homocisteína em fatias de cortex cerebral e coração e em cultura de astrócitos de ratos adultos. Seguindo esses modelos experimentais, investigamos alguns parâmetros bioquímicos em córtex cerebral e coração de ratos. Posteriormente foi analisado o efeito protetor do calcitriol. No primeiro capítulo da presente tese, observamos que a incubação de 30 μM de Hcy por 30 min e 60 min em fatias de coração, alterou a função e a permeabilidade mitocondrial, o estado redox e a atividade das enzimas da cadeia respiratória; o calcitriol foi capaz de prevenir a maioria dos efeitos da Hcy. No segundo capítulo, vimos que em fatias de córtex cerebral a Hcy prejudica o metabolismo energético, aumentando a morte neuronal e induzindo estresse oxidativo. Todavia, o calcitriol atenuou esses efeitos deletérios induzidos pela Hcy através da ativação do receptor de vitamina D. No último capítulo desta tese, realizamos um estudo em cultura primária de astrócitos corticais de ratos Wistar adultos. Nossos resultados demonstram que a Hcy ativa a via do fator nuclear kappa B (NFκB), inibindo a expressão de heme oxigenase 1 (HO-1), promovendo alterações morfológicas, aumentando a resposta inflamatória e diminuindo as defesas antioxidantes e a atividade da Na$^+$, K$^+$ - ATPase. Em resumo, em todos modelos experimentais estudados nesta tese, a Hcy, mesmo em concentrações leves e moderadas causou alterações na homeostasia celular. A vitamina D preveniu parte destes efeitos, tornando-se um possível ferramenta terapêutica no intuito de atenuar os efeitos da Hcy.

ABSTRACT

Hyperhomocysteinemia (HHcy), mild and moderate are a risk factor for cardiovascular and cerebral diseases, but the mechanisms and complications of this condition are not yet well established. It occurs in 5-10% of the general population and 40% of patients with peripheral vascular and cerebrovascular disease. Recent studies have shown that vitamin D (calcitriol) has protective effects in various experimental models which emphasize their potential antioxidant actions. The main objective of this PhD thesis was to establish an experimental with different concentrations of homocysteine in slices of cerebral cortex and heart in adult rat astrocyte cultures. Following this experimental model, we investigated some biochemical parameters in the cerebral cortex and heart of rats. It was subsequently examined the protective effect of calcitriol. In the first chapter of this thesis, we found that incubation of 30 µM of Hcy for 30 min and 60 min in heart slices change the function and mitochondrial permeability, redox state and activity of the enzymes of the respiratory chain; calcitriol was able to prevent most of the effects of Hcy. In the second chapter, we demonstrated that Hcy in the cerebral cortex slices impairs energy metabolism, increasing neuronal death and inducing oxidative stress. However, calcitriol attenuated these Hcy-induced deleterious by activation of vitamin receptor D. In the last chapter of this thesis, we conducted a study in primary culture of cortical astrocytes. Our results demonstrate that the Hcy active the pathway of nuclear factor kappa B (NFκB) inhibiting heme oxygenase expression 1 (HO-1), promoting morphological changes, increasing the inflammatory response and decreased antioxidant defenses and activity of the Na +, K + - ATPase. In summary, in all experimental models studied in this PhD thesis, Hcy, even in mild and moderate concentrations caused deleterious actions in cellular homeostasis. Vitamin D warned of these effects, becoming a potential therapeutic target in order to attenuate the effects of Hcy.

LISTA DE ABREVIATURAS E FÓRMULAS

$1,25(OH)_2D_3$: 1,25 dihidroxivitamina D $_3$

1,25 dihidroxicolecalciferol: Calcitriol

$24,25(OH)_2D_3$: 24,25-desidroxivitamina D

$25(OH)D$: 25-hidroxivitamina D

5-MeTHF: 5-metiltetraidrofolato

O_2: Oxigenio singlet

ATP: Trifosfato de adenosina

BHMT - Betaina homocisteina metiltransferase

CAT: Catalase

CBS: Cistationina β-sintase

CGL: cistationina γ-liase

CO: Monóxido de carbono

DCF: 2'7'diclorofluoresceína

DNA: ácido desoxirribonucleico

EIM: Erro inato do metabolismo

ERN: Espécies reativas de nitrogênio

ERO: Espécies reativas de oxigênio

GPx: Glutationa peroxidase

GSH: glutationa (forma reduzida)

GSSG: Dissulfeto de glutationa

H_2O_2: Peróxido de hidrogênio

Hcy: Homocisteina

HHcy: Hiperhomocisteinemia

HO-1: Heme oxigenase 1

IFN-γ: Interferon gamma

IL-1β: Interleucina 1-β

IL-6: Interleucina 6

IL-8: Interleucina 8

LPS: Lipopolissacarídeo

MAT: metionina adenosiltransferase

MCP-1: Proteína quimiotática de monócito do tipo 1

Met: Metionina

MS: metionina sintase

MTHFR: Metileno tetrahidrofolato reductase

NFκB - Fator Nuclear kappa Beta

NMDA: N-metil-*D*-aspartato

NO: óxido nítrico

Nrf2: Fator Nuclear Eritroide 2

$O_2^{\bullet-}$: Ânion superóxido

OCl^-: Ânion hipoclorito

$OH^\bullet$: Radical hidroxila

PGE_2: Prostaglandinas

PLP: Piridoxal fosfato

PTH: Paratormônio

SAH: S-adenosil homocisteína

SAHH: S-adenosil homocisteína hidrolase

SAM: S-adenosilmetionina

SNC: Sistema nervoso central

SOD: Superóxido dismutase

TBARS: substâncias reativas ao ácido tiobarbitúrico

TNF-α : Fator de necrose tumoral alfa

UVB: Raios ultravioleta B

VDR: Receptores de vitamina D

Vitamina D3: colecalciferol

LISTA DE FIGURAS

1.1. Homocisteína

A Homocisteína (Hcy) é um aminoácido sulfurado não protéico e foi identificado pela primeira vez por De Vigneaud em 1932, ela atua como um intermediário no metabolismo da metionina (Met). A Met é obtida a partir da dieta e da degradação de proteínas endógenas e tem um papel fundamental nos processos de metilação moleculares. Quando é convertida à S-adenosilmetionina (SAM) pela enzima metionina adenosiltransferase (MAT, EC 2.5.1.6) a Met recebe um grupo adenosil do trifosfato de adenosina (ATP). A SAM é o principal doador de grupos metil presente no meio biológico, ela é convertida à S-adenosil homocisteína (SAH) por diversas metiltransferases, que são específicas para cada substrato. A SAH pode ser hidrolisada a Hcy e adenosina pela S-adenosil homocisteína hidrolase (SAHH, EC 3.3.1.1) (Williams e Schalinske, 2010).

A partir deste ponto, o catabolismo da Hcy pode seguir duas vias: (1) a remetilação à Met, onde a Hcy recebe um grupo metil proveniente do N5-metiltetraidrofolato, através da reação catalisada pela metionina sintase (MS, EC 2.1.1.13), ou da betaína, através da reação catalisada pela betaína homocisteína metiltransferase (BHMT, EC 2.1.1.15), ou ainda (2) a transulfuração à cisteína, onde a Hcy é condensada com a serina, produzindo cistationina, através da reação catalisada pela enzima cistationina β-sintase (CBS; EC 4.2.1.22), que utiliza como coenzima o piridoxal fosfato (PLP), no passo seguinte, a enzima cistationina γ-liase (CGL, EC, 4.4.1.1) promove uma clivagem formando a cisteína (Figura 1). A rota de remetilação é amplamente distribuída no organismo, enquanto que a via de transulfuração da Hcy tem distribuição

limitada, participando do catabolismo da Hcy principalmente no fígado, rins, intestino delgado, pâncreas e cérebro (Khanal et al., 2011; Stipanuk e Ueki, 2011; Ajith e Ranimenon, 2015).

O fígado é o principal órgão de degradação do excesso de Met e manutenção dos níveis adequados de Hcy. A SAM tem um papel importante na regulação dos níveis de Hcy, pois quando os níveis de SAM estão elevados, existe uma redução na via de remetilação da Hcy à Met através da inibição da atividade da metileno tetrahidrofolato redutase (MTHFR). Por outro lado, quando ocorre o acúmulo de Hcy, a SAM promove o catabolismo desse aminoácido através da transulfuração por ativação da cistationina β-sintase (Paul e Borah, 2015).
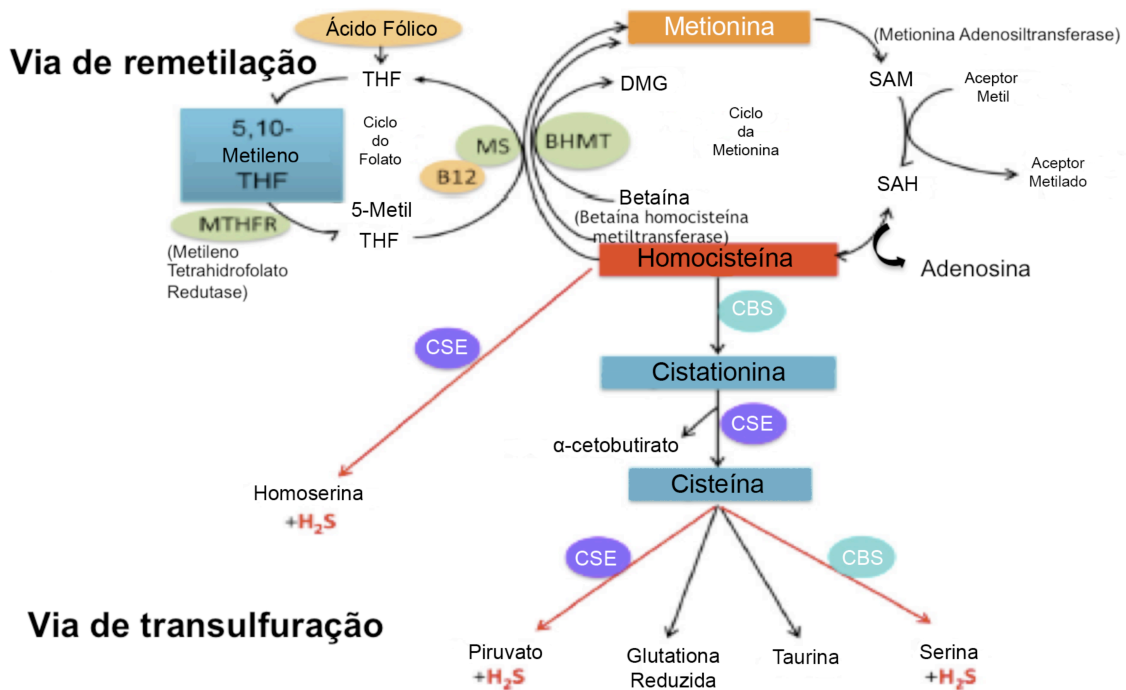
Figura 1: Metabolismo da homocisteína. SAM: S-adenosilmetionina; SAH: S-adenosil homocisteína; CBS: cistationina β-sintase; CSE: cistationina γ-liase; MS: metionina sintase; BHMT: betaína homocisteína metiltransferase; MTHFR: metileno tetrahidrofolato redutase; DMG: dimetil glicina; THF: tetrahidrofolato; 5-10-MTHF: 5,10-metilenotetrahidrofolato; 5-Me-THF: 5-metiltetrahidrofolato (Adaptado de Li, 2015).

1.1.1. Hiperhomocisteinemia

Em indivíduos normais, níveis plasmáticos de Hcy variam entre 5 a 15 µmol/L. A HHcy, condição gerada pela elevação dos níveis de Hcy, pode ser causada por fatores genéticos e ambientais e é classificada em três graus conforme a severidade: leve (16-30 µmol/L), moderada (31-100 µmol/L) e severa (> 100 µmol/L) de Hcy (Banecka-Majkutewicz et al., 2012). A HHcy severa é rara e pode ser causada por deficiência genética na atividade da enzima CBS caracterizando a homocistinuria (HCU) (Kolling et al., 2014), ao contrário da HHcy leve e/ou moderada, que são mais prevalentes na população.

A HHcy leve e moderada, que é o foco dessa tese, pode ser causada por deficiências nutricionais do ácido fólico, vitaminas B_6 e B_{12}, ingestão aumentada de Met, insuficiência renal crônica, uso de alguns medicamentos, como por exemplo, metotrexato, L-dopa, fenitoína e carbamazepina, idade e sexo (Scherer et al., 2011; Ajith e Ranimenon, 2015).

1.1.2. Toxicidade da Hiperhomocisteinemia

A toxicidade da Hcy se deve principalmente a sua capacidade de ser captada através de um transportador específico de membrana, entretanto a ausência de vias de eliminação da Hcy no cérebro, também favorece sua toxicidade, uma vez que a enzima betaína homocisteína metiltransferase (BHMT) e parte da via de transulfuração até cisteína não estão presentes no cérebro (Finkelstein, 2007; Obeid, 2013).

Sabe-se que a Hcy aumenta a neurotoxicidade do peptídeo beta-amilóide por indução de estresse oxidativo (Ho et al., 2001). Outros trabalhos mostram que a HHcy leve diminui a captação de glutamato, a atividade da Na^+, K^+-ATPase, induz o estresse oxidativo em córtex cerebral de ratos (Scherer et al.,

2013). Resultados obtidos pelo nosso grupo, mostram também que a HHcy severa promove peroxidação lipídica e aumenta a produção de espécies reativas de oxigênio (ERO), além de diminuir as defesas antioxidantes enzimáticas e os níveis de nitritos em coração de ratos (Kolling et al., 2011), bem como diminui a captação de glutamato em hipocampo de ratos (Machado et al., 2011).

Já em relação aos danos vasculares, a Hcy aguda reduz a biodisponibilidade de óxido nítrico (NO) aumentando a adesão e agregação plaquetária, estimulando a formação de trombos, além de alterar a morfologia vascular e estimular a inflamação (da Cunha et al., 2011). Ela também pode promover um estado pró-inflamatório, verificado através de estudos *in vitro* que demonstraram que a Hcy é capaz de induzir um aumento na expressão de várias citocinas pró-inflamatórias em tecidos e cultura de células (Stanger et al., 2004; Lee et al., 2005; Julve et al., 2013).

1.2. Ações teciduais e celulares da Hiperhomocisteinemia

1.2.1. Sistema Nervoso Central e Cardiovascular

Estudos têm demonstrado que a HHcy leve é um fator de risco para doenças cerebrais como Parkinson (dos Santos et al., 2009), Alzheimer (Minagawa et al., 2010), depressão e esquizofrenia (Permoda-Osip et al., 2013; Wysokinski e kloszewska, 2013), bem como cardíacas, entre elas aterosclerose e tromboembolismo (Den Heijer et al., 2005; Abbracchio et al., 2006). Uma elevação de cerca de 5μmol/L nos níveis plasmáticos de Hcy está associado com o desenvolvimento de doenças cardíacas (Xie et al., 2015). Pacientes com doença de Parkinson apresentam níveis de Hcy 30% maiores no plasma do que indivíduos normais (dos Santos et al., 2009).

Os efeitos tóxicos da Hcy ainda não estão bem elucidados em relação aos mecanismos de ação. A HHcy severa tem sido evidenciada em pacientes acometidos por doenças neuromusculares (Huang et al., 2011; Leishear et al., 2012), neurodegenerativas (Timkova et al., 2016), psiquiátricas (Pana, 2015; Bottiglieri, 2005), vasculares (Faraci e Lentz, 2004), hepáticas (Adinolfi et al., 2005) e pulmonares (Jiang et al., 2005; Hamelet et al., 2007). No SNC, a Hcy está bem associada à morte neuronal via exicitotoxidade, através da ativação de receptores glutamatérgicos metabotrópicos do grupo I (Zieminska et al., 2003) e ionotrópicos N-metil-*D*-aspartato (NMDA) (Timkova et al., 2016). A Hcy também pode danificar células (Perna et al., 2003; Weiss et al., 2003; Zou e Benerjee, 2005) por auto-oxidação do seu grupo tiol, causando prejuízo à homeostase redox. Ela pode também causar prejuízo nos processos de metilação, onde um acúmulo de Hcy leva a um aumento na SAH, que é um potente inibidor da reação de metilação. Estes são vitais para a função neurológica como a metilação de aminas biogênicas, da mielina e síntese de fosfatidilcolina (Troen, 2005).

1.2.2. Astrócitos

Astrócitos são células gliais, abundantes no SNC que representam 50% do número total de células, com características fenotípicas e de citoarquitetura única que, idealmente, os posicionam a sentir e responder a mudanças no seu microambiente. Eles estendem numerosos processos astrocíticos formando domínios anatômicos altamente organizados com poucas sobreposições entre células adjacentes e são interconectados em redes funcionais através de junções gap (Kirchhoff et al., 2001). Alguns destes processos estão em contato

com vasos sanguíneos intraparenquimais através de processos especializados chamados pés terminais (pés astrocíticos), enquando outros expressam uma ampla gama de receptores e canais iônicos que circundam as sinapses. Desta maneira, os astrócitos apresentam um papel importante no acoplamento neurometabólico e neurovascular (Allaman et al., 2011). Eles apresentam uma morfologia heterogênea sendo dividida em duas subpopulações: astrócitos fibrosos, que são encontrados na substância branca e apresentam menos ramificações e extensões cilíndricas e os astrócitos apresentam uma localização na substância cinzenta e possuem muitos processos ramificados que envolvem as sinapses e os pés terminais que envolvem os vasos sanguíneos (Wang e Bordey, 2008).

Existem pouco trabalhos na literatura demonstrando a toxicidade da Hcy em astrócitos. Loureiro et al., (2010) em seu estudo, utilizando um modelo co-cultivo celular neurônio/astrócito, observou que o citoesqueleto de astrócitos corticais e não dos neurônios foi alvo da toxicidade da Hcy, e que este efeito foi mediado por sinalização redox. Foi demonstrado ainda que os astrócitos foram capazes de sobreviver a uma concentração de 100 μM de Hcy, reorganizando seu citoesqueleto e protegendo os neurônios da toxicidade da Hcy (Loureiro et al., 2010).

1.3. Hiperhomocisteinemia leve e Modelos experimentais

Para indução das HHcy leve e moderada podem ser utilizados animais com deficiência genética heterozigótica na CBS (Dayal et al., 2004) ou na MTHFR (Chen et al., 2001) e principalmente intervenções dietéticas que afetam o metabolismo da Hcy via transulfuração ou remetilação (Blaise et al., 2007).

Uma deficiência de vitamina B_6 limita o fluxo da Hcy via transulfuração, enquanto que a deficiência de vitamina B_{12} ou ácido fólico prejudica a remetilação da Hcy à Met. Recentemente, nosso grupo desenvolveu um modelo experimental *in vivo* de HHcy leve crônica, onde os ratos são submetidos a 2 injeções subcutâneas diárias de Hcy (0,03 μmol/g de peso corporal) por 30 dias (Scherer et al., 2011). Utilizando este modelo Scherer et al., (2011) mostrou que a HHcy leve induz o estresse oxidativo e a inflamação em córtex cerebral de ratos (Scherer et al., 2012).

1.4. Estresse oxidativo

Os radicais livres são produzidos como uma função fisiológica do metabolismo celular, entretanto quando em excesso, podem reagir com moléculas como lipídios e proteínas, provocando a transferência de um ou mais eletrón da molécula para o radical livre, o que acaba produzindo uma nova molécula instável. Alguns fatores como o tabagismo, poluentes ambientais, radiação, drogas, pesticidas, solventes industriais e ozônio também ajudam a promover a produção de radicais livres (Lobo et al., 2010; Carocho e Ferreira, 2013). Todo esse processo de oxidação danifica moléculas aterando conformações e funções, criando assim uma reação em cadeia prolongando o dano oxidativo e estas lesões ocasionadas podem levar ao estresse oxidativo (Gutowski et al., 2013).

O estresse oxidativo pode ser causado de duas maneiras: I) através da modificação direta de macromoléculas intra/extracelulares que alteram suas funções, levando a efeitos patológicos, II) e por alterações no estado redox de moléculas, modificando o efeito na tradução de sinais, levando a aumentos ou

diminuições de sinalização e isso leva ao comprometimento do funcionamento celular e/ou morte celular (Lenaz, 2012; Weinreb et al., 2013).

O dano oxidativo ocorre muito rápido devido a meia-vida dos radicais livres ser muito curto e ter uma alta reatividade (Halliwell, 2007). Organismos aeróbicos precisam conviver com espécies reativas de oxigênio produzidos por eles mesmos, e concomitantemente equilibrar suas concentrações de radicais livres abaixo de níveis tóxicos através de enzimas antioxidantes (superoxido distmutase - SOD, catalase - CAT e glutationa peroxidase - GPx) e antioxidantes não enzimáticos (vitamina C, glutationa, entre outros) (Kalyanaraman et al., 2013).

Para quantificar diretamente os radicais livres e os danos oxidativos associados, usa-se biomarcadores, substâncias que podem ser mensuradas e predizer a incidência de um acontecimento ou doença. Esses são relacionados ao estresse oxidativo e são mais estáveis que as espécies reativas de oxigênio e nitrogênio (ERN). ERO e ERN podem ser benéficas ou deletérias para estes sistemas vivos. Em baixas concentrações atuam na defesa contra agentes infecciosos e nos processos de sinalização intracelular (Halliwell e Gutteridge, 2007; Halliwell, 2011; Gutowski e Kowalczyk, 2013).

Para avaliar a produção de especies reativas, de maneira indireta, utilizam-se medidas de reatividade e de altareatividade de enzimas e sistemas antioxidantes (Birben et al., 2012). Portanto esse equilíbrio entre a produção e a neutralização de espécies reativas por antioxidantes é muito importante, e se esse equilíbrio pender para a superprodução de espécies reativas, as células sofrem consequências a insultos oxidativos (Halliwell, 2011).

As espécies reativas mais importantes são o ânion superóxido ($O_2\bullet-$), peróxido de hidrogênio (H_2O_2), radical hidroxila (OH•), ânion hipoclorito (OCl^-) e o oxigênio "singlet" (O_2), e eles são gerados nos sistemas vivos através do metabolismo energético celular. A redução tetravalente completa do oxigênio molecular (O_2) na cadeia transportadora de elétrons mitocondrial é essencial para a formação de adenosina trifosfato (ATP) e aproximadamente 5% do O_2 não é completamente reduzido à água nesse processo, sendo convertido a $O_2^{.}$, H_2O_2 e $OH^{.}$. O $NO^{.}$ e o peroxinitrito ($ONOO^-$), formado a partir da reação do $NO^{.}$ com o $O_2^{.-}$, constituem as principais ERN. A GPx, localizada nas membranas celulares, decompõe o H_2O_2 através do acoplamento de sua redução a H_2O com a concomitante oxidação da glutationa (GSH) ao dissulfeto de glutationa (GSSG) ($H_2O_2 + 2GSH \rightarrow GSSG + 2\ H_2O$) (Halliwell e Gutteridge, 2007; Halliwell, 2011; Gutowski e Kowalczyk, 2013).

1.5. Na$^+$, K$^+$-ATPase

A Na$^+$, K$^+$-ATPase é altamente vulnerável aos insultos oxidativos e pode ser inibida pelos radicais livres (Wang e cols., 2014). Essa enzima é muito importante para funções celulares e sinápticas. Ela é responsável pela manutenção do gradiente iônico neural, através do transporte ativo de três íons de Na$^+$ para o meio extracelular e dois íons de K$^+$ para o meio intracelular com concomitante hidrólise de ATP, sendo que a mesma consome em torno de 60% do ATP formado pelo cérebro. Essa enzima oligomérica é composta de duas subunidades α, que contém os sítios de ligação para os íons Na$^+$ e K$^+$, ATP e glicosídios, e duas subunidades β, na forma de glicoproteínas (Scherer et al., 2013).

Estudos realizados no nosso grupo de pesquisa mostraram que a HHcy leve aumenta a produção de espécies reativas (Scherer et al., 2011) e diminui a atividade e o imunoconteúdo da Na$^+$, K$^+$-ATPase (Scherer et al., 2013). Essas alterações na atividade da Na$^+$,K$^+$-ATPase têm sido associadas a diversas patologias que afetam o SNC, tais como enxaqueca (Suhail, 2010), doença de Alzheimer (Vitvitsky et al., 2012), Parkinson e epilepsia (Benarroch, 2011). Além disso, estudos prévios realizados em nosso grupo de pesquisa mostraram que alguns aminoácidos como a Met (Stefanello et al., 2011), prolina (Ferreira et al., 2011) e a Hcy (Machado et al., 2011) inibem a atividade da Na$^+$,K$^+$-ATPase cerebral, provavelmente, através da indução de estresse oxidativo.

1.6. Inflamação

A inflamação é um mecanismo homeostático utilizado para proteger a integridade do organismo contra agentes nocivos endógenos ou exógenos. A resposta inflamatória envolve células e fatores solúveis, libera mediadores e recruta leucócitos da circulação, que se ativam no local da inflamação e liberam mais mediadores. Os mediadores são conhecidos como proteínas de fase aguda (proteína C reativa, fibrinogênio, alfa-1-glicoproteína ácida, alfa-1-antitripsina, haptoglobina e ceruloplasmina), interleucinas [interleucina 1-β (IL-1β), interferon gamma (IFN-γ) e fator de necrose tumoral alfa (TNF-α), quimiocinas (interleucina 8 (IL-8) e proteína quimiotática de monócito do tipo 1 (MCP-1) prostaglandinas (PGE$_2$) e NO$^·$ (Saleron et al., 2002; Zhang, 2008; Colton, 2009).

As citocinas pró-inflamatórias TNF-α, IL-1β e interleucina 6 (IL-6) desempenham papel crucial na resposta de fase aguda. O TNF-α, secretado por macrófagos, linfócitos e monócitos (Tourjman et al., 2013), é um potente ativador

de neutrófilos. Além disso, essa citocina estimula as células endoteliais a expressar moléculas de adesão e promove alterações na permeabilidade vascular (Wolf et al., 2013). A IL-6 é produzida em resposta ao TNF-α, e a IL-1β é produzida por macrófagos, neutrófilos, células epiteliais e endoteliais em resposta a outras citocinas, como o TNF-α, ou produtos bacterianos como o lipopolissacarídeo (LPS). Ela atua sobre o endotélio aumentando a expressão de moléculas de adesão (Cao et al., 2005; Kondera-Anasz et al., 2005), ativando as fibras aferentes do nervo vago, as quais servem de sensor para a inflamação, transmitindo ao SNC o qual estimula o nervo vago eferente para a produção de ACh, que induz então a inibição da síntese e liberação de citocinas pró-inflamatórias por macrófagos e outras células produtoras de citocinas (Pavlov e Tracey, 2012).

Já foi demonstrado que a Hcy ativa células endoteliais em cultura, o que resulta no aumento da expressão de quimiocinas e moléculas de adesão (Hohsfield e Humpel, 2010). Poddar et al. (2001) demonstrou que concentrações de baixas de Hcy promovem um estado pró-inflamatório com o aumento da expressão e secreção de IL-8 e MCP-1 em cultura de células endoteliais aórticas humanas. E em altas concentrações, ela aumenta a concentração das citocinas pro-inflamatorias (TNF-α e IL-1β), em cérebro de ratos (da Cunha et al., 2012; Scherer et al., 2014).

1.7. Vias de Sinalização

1.7.1. Fator nuclear kappa B

O Fator nuclear kappa B (NFκB) pertence a uma família de fatores de transcrição diméricos responsáveis pela coordenação das respostas

inflamatórias; imunidade inata e adaptativa; e diferenciação, proliferação e sobrevivência celular em quase todos os organismos celulares (Gerondakis et al., 2006; Vallabhapurapu e Karin 2009; Hayden e Ghosh 2012). Em mamíferos, a família do NFκB consiste de cinco monômeros (subunidades - p65/RelA, RelB, cRel, p50, and p52) que formam homodímeros ou heterodímeros que ligam-se ao DNA diferentemente e são regulados por duas vias: a clássica (via canônica), que está relacionada à expressão de genes associados à inflamação, à resposta imunológica inata, à anti-apoptose e à sobrevivência celular (Xiao, 2004); e a via alternativa (não-canônica) que está associada à expressão de genes que atuam no desenvolvimento e manutenção de órgãos linfóides secundários (Alcamo et al., 2002).

Na via clássica, para que o NFκB seja ativado, o Iκ B é fosforilado no resíduo de serina pelo complexo de proteína quinase IKK. Essa fosforilação é o sinal para a ubiquitinação e posterior degradação do Iκ B pelo proteassoma. No citoplasma um conjunto de proteínas adaptadoras e ancoradoras (TRAFs, MyD88 e TIRAP) e quinases (RIP, IRAK) formam um complexo quando há um estímulo, facilitando o recrutamento da IKK. Após a degradação do IκB os dímeros do NFκB (p50-p65) são liberados e migram para o núcleo onde atuarão na regulação da transcrição de genes específicos (Kaltschmidt et al., 2005).

O complexo IKK contém duas subunidades catalíticas denominadas IKKα (IKK1) e IKKβ (IKK2) e uma subunidade não catalítica denominada NEMO (do inglês NF-κB essential modulator). Existem diversas combinações entre as subunidades do IKK, podendo ser homo ou heterodímeros de IKKα ou IKKβ , associados ou não ao NEMO (Mercurio et al., 1999).

O NFκB é expresso no SNC e periférico, tanto por neurônios quanto por

células da glia. Diversos dímeros já foram descritos no SNC, porém os mais comuns são p50-p65 e p50-p50 (O'Neill e Kaltschmidt, 1997). Em condições não patológicas a ativação do NFκB pode estar envolvida na plasticidade, desenvolvimento neural e na atividade sináptica.

1.7.2. HO-1

A heme oxigenase (HO) catalisa o primeiro e limitante passo da degradação enzimática do heme, produzindo monóxido de carbono (CO), ferro e biliverdina, que é convertida a bilirrubina através da biliverdina redutase (Ryter et al., 2006; Haines et al., 2012). Atualmente, são conhecidas duas isoenzimas geneticamente distintas de HO, HO-1 e HO-2. A HO-2 representa a isoforma constitutiva não-induzível que é expressa principalmente nas células neurais e podem atuar como antioxidantes (Syapin, 2008). Por outro lado, a forma induzível (HO-1), que apresenta baixos níveis de expressão basal na maioria das células e tecidos, é *super-regulada* por uma grande variedade de estímulos de estresse oxidativo. Onde seu grupamento heme livre pode ser originado de fontes extracelulares, na degradação de hemoglobima, e de fontes intracelulares, a partir do metabolismo de proteínas que contenham o heme em sua estrutura. E assim, em situações de excitotoxidade, ocorre um aumento da produção de heme em sua forma livre e logo após sua degradação pela HO, por que ele não pode ser degradado. Devido ao seu padrão de regulação, a indução de HO-1 tem sido geralmente considerada como sendo uma resposta celular adaptativa contra a toxicidade do estresse oxidativo (Vile et al., 1994; Doré et al., 1999).

Recentemente, estudos têm relatado que a HO-1 possui funções imunomodulatórias e anti-inflamatórias (Gozzelino et al., 2010). Essa ligação potencial entre HO-1 e inflamação foi inicialmente demonstrada em modelos animais, onde a *super-regulação* de HO-1 atenuou os efeitos pró-inflamatórios (Willis et al., 1996). Alem disso, a produção de HO-1 é regulada pelo fator de transcrição Fator Nuclear Eritroide 2 (Nrf2), levando a síntese de enzimas do sistema de defesa antioxidante. A HO-1 pode atuar sequestrando NO, inibindo a síntese da iNOS, contribuindo no controle do estresse oxidativo (Wakabayashi et al., 2010).

1.8. Metabolismo da Vitamina D

A vitamina D é um composto orgânico lipossolúvel, considerado um hormônio esteróide, essencial para o metabolismo dos seres vivos. Seu papel principal está relacionado à manutenção da homeostase do cálcio e fósforo (Kriebitzsch et al., 2011). A descoberta da participação da vitamina D na homeostase do sistema imunológico, na existência de receptores de vitamina D (VDR) em vários tecidos e células e também na capacidade de transformar a 25-hidroxivitamina D [25(OH)D] no metabólito ativo, 1,25 dihidroxivitamina D_3 ($1,25(OH)_2D_3$) vem sendo discutida.

A forma ativa da vitamina D pode ser formada principalmente a partir da via endógena (Figura 2) onde o 7-desidrocolesterol localizado na pele é transformado em colecalciferol (vitamina D3) pela ação de raios ultravioleta B (UVB) através de termo-reação.

14
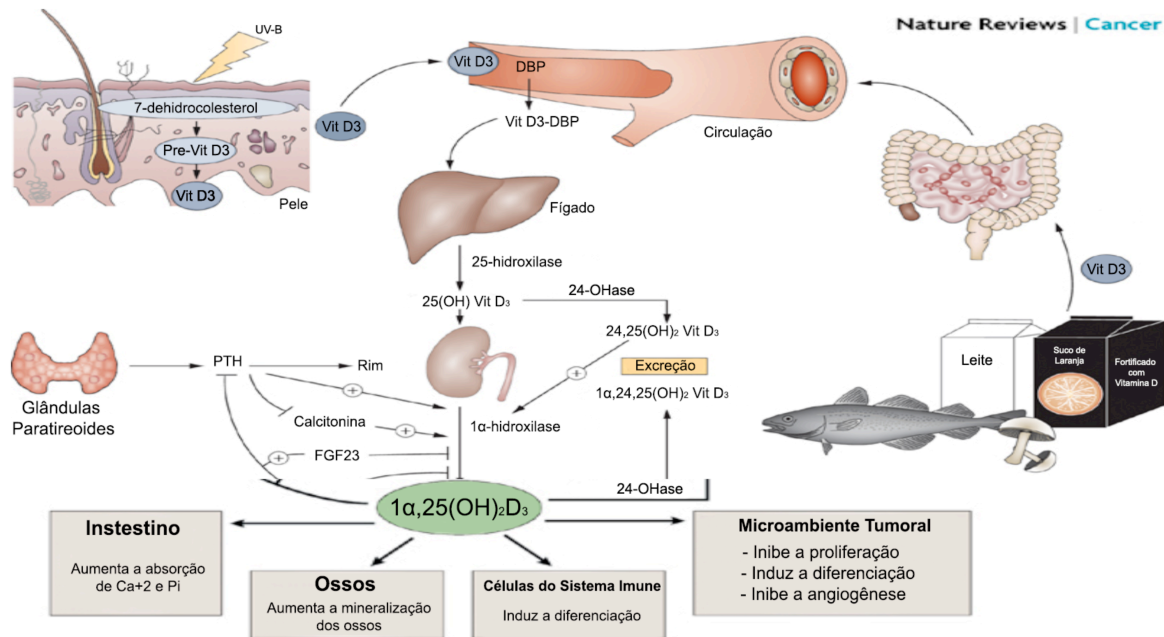
Figura 2: Metabolismo da vitamina D: A vitamina D_3 é formada a partir de 7-desidrocolesterol na pele sob a influência da radiação UVB ou é obtido a partir de fontes dietéticas por absorção através do intestino. A vitamina D_3 circulante é transportado por uma proteina ligante de vitamina D. No fígado, a vitamina D_3 é convertido para 25(OH)D pela 25-hidroxilase. A 25(OH)D é então convertido para o metabolito ativo da vitamina D, $1,25(OH)_2D_3$ no rim pela enzima 1α-hidroxilase. 1α-hidroxilase é regulada por vários mecanismos, incluindo PTH, calcitonina, e FGF23. Vitamina D ativa pode ser desativada por adição de outro grupo hidroxil (OH) na posição 24, após o que o produto final é excretado. (Adaptado de Kristin et al., 2015).

A exposição excessiva a radiação UVB não causa intoxicação por vitamina D, pois ela mesma leva a degradação do 7-desidrocolesterol e do colecalciferol (Latimer et al., 2014; Lin, 2016). E a outra via para produção de vitamina D é a via exógena (Figura 2) que ocorre pela ingestão de precursores encontrados em certos alimentos, como peixe de mares profundos, de origem animal (vitamina D_3), alguns grãos e plantas, de origem vegetal (vitamina D_2) e a gema de ovo (Vitamina D_2 e D_3), onde são absorvidos via intestinal e incorporado aos quilomicrons para serem transportados pelo sistema linfático até a circulação venosa (Latimer et al., 2014; Goldmith, 2015; Wintermeyer et al., 2016). Aproximadamente 80% da vitamina D ativa vem da via endógena, que é dependente da exposição solar e dos níveis cutâneos de pigmentação

melanocítica, e apenas, 20% das necessidades corporais diárias são supridas pela alimentação e assim, diferenciam a vitamina D das demais vitaminas, cuja principal fonte é a dieta (Huotari e Herzig, 2008).

O colecalciferol e o ergocalciferol são absorvidos pelo intestino delgado e também são transpostrados para o fígado por uma proteína ligante de vitamina D e em menor quantidade pela albumina (Ritterhouse, 2011), onde sofre hidroxilação para 25(OH)D, o fígado é seu principal reservatório e ela é o principal metabolito circulante (Wintermeyer et al., 2016). Nos rins, a 25(OH)D é hidroxilada, novamente, pela enzima 1α-hidroxilase a $1,25(OH)_2D_3$, também conhecida como calcitriol, que é o produto mais ativo, uma vez sintetizado, é transportado na circulação até os órgãos e tecidos onde irá exercer suas funções. Essa produção é regulada pelo paratormônio (PTH) e pelos níveis séricos de cálcio e fósforo (Querales et al., 2010; Dougherty, 2016). E uma pequena parcela é hidroxilada a 24,25-desidroxivitamina D $[24,25(OH)_2D_3]$, pela enzima α-hidroxilase, que é um metabólito hidrossolúvel inativo, também conhecido como ácido calcitróico, excretado na bile (Querales at al., 2010; Wintermeyer et al., 2016).

O aumento dos precursores de vitamina D resulta no acúmulo de 25(OH)D no fígado, aumentando a produção de 24,24-diidroxicolecalciferol pela ação da 24-hidroxilase, e dessa forma, aumentando a absorção de cálcio no intestino. Já nos períodos de menor produção de vitamina D, a 25(OH)D é mobilizada e transformada em $1,25(OH)_2D$ (Karras et al., 2016; Wintermeyer et al., 2016). Diante disso, a 1α-hidroxilase é descrita em vários tecidos (próstata, pulmão, células do sistema imune), tornando possível a produção de vitamina D ativa (Vuolo et al., 2012). E assim, a presença do VDR em células do trato

reprodutivo, sistema endocrinológico, musculatura lisa e esquelética, coração pele, cérebro e fígado sugere a participação da vitamina D em outras funções regulatórias (Eliades e Spyrou, 2015).

A 25-hidroxivitamina D e a 1α-hidroxilase estão presentes em muitos tecidos, onde encontra-se também o VDR ao qual se liga a 1,25(OH)$_2$D. Após ela exercer seus efeitos nos tecidos, é degradada pelo estímulo da expressão do gene CYP24 de forma a não entrar na corrente sanguínea e interferir no metabolismo do cálcio (Gezen-AK et al., 2013; Wintermeyer et al., 2016; Lin, 2016).

1.8.1. Ação protetora da vitamina D

Dentre as várias funções da vitamina D, podemos relatar que o calcitriol, já liberado, que liga-se aos VDR, amplamente distribuídos no organismo, podendo então estimular a síntese de algumas proteínas relacionadas à regulação do metabolismo de cálcio e fósforo, modular a atividade do sistema imune, síntese e secreção de insulina, proliferação e diferenciação celular, apoptose e angiogênese (Sen e Chakraborty, 2011). Desta forma, ela é capaz de diminuir a proliferação celular tanto nas células cancerosas, como nas células normais, e induzir a diferenciação celular. Estas ações da vitamina D poderiam ser uma explicação plausível para a diminuição do risco de morte nos pacientes com câncer que apresentam níveis altos de 25(OH)D (Kimmie, 2014).

Podemos destacar também as ações da vitamina D no SNC, onde ela pode ser chamada de *"neuro-esteróide" (*Kocovska et al., 2012), pois estruturas encefálicas como o córtex cerebral e hipocampo, expressam o VDR e a enzima 1α-hidroxilase e assim sintetizam a 1,25(OH)$_2$D (Lardner, 2015). Já foi descrito

na literatura que a vitamina D é capaz de proteger neurônios contra a toxicidade da proteína beta-amilóide e prevenir danos oxidativos (Banerjee et al., 2015). Podemos ressaltar também que a adequada exposição solar está bem associada a menor ocorrência de doenças cardiovasculares (Mandarino et al., 2015).

2.1. Objetivo Geral

Considerando que há uma estreita relação entre a HHcy leve e moderada e o desenvolvimento de doenças cardiovasculares e cerebrais e que a fisiopatologia dessas doenças está ligada fortemente com as perturbações bioenergéticas, oxidativas e inflamatórias, o presente trabalho teve como objetivo avaliar: parâmetros de metabolismo energético, incluindo a análise da função mitocondrial; parâmetros de estresse oxidativo e inflamatórios em fatias de córtex cerebral e coração de ratos, bem como investigar parâmetros como viabilidade celular, atividade da Na^+,K^+-ATPase, defesas antioxidantes e resposta inflamatória em cultura cortical de astrócitos de ratos adultos submetidos a diferentes concentrações de Hcy. Também investigamos o possível papel protetor da vitamina D sobre as alterações bioquímicas observadas no modelo experimental utilizado.

2.2. Objetivos específicos:

Os objetivos específicos estão subdivididos em três capítulos, que serão apresentados na forma de artigos científicos, como segue:

Capítulo I

- Avaliar a atividade dos complexos da cadeia respiratória, bem como a função mitocondrial através da massa e potencial;
- Determinar os parâmetros de estresse oxidativo, bem como o imunoconteúdo do receptor de vitamina D em fatias de coração de animais submetidos a um modelo experimental de HHcy leve.

- Investigar o possível papel protetor do pré tratamento de vitamina D sobre as alterações bioquímicas encontradas.

Capítulo II

- Medir os parâmetros de estresse oxidativo, bem como o imunoconteúdo do receptor de vitamina D em fatias de córtex cerebral de animais submetidos ao modelo experimental de HHcy leve;

- Verificar a atividade dos complexos da cadeia respiratória, bem como a função mitocondrial através da massa e potencial;

- Investigar o possível papel protetor do pré tratamento de vitamina D sobre as alterações bioquímicas encontradas.

Capítulo III

- Examinar o efeito da Hcy sobre a viabilidade mitocondrial, e observar alterações no citoesqueleto e na expressão de marcadores gliais em cultura primária de astrócitos de ratos adultos;

- Investigar o efeito da Hcy sobre a atividade da Na^+,K^+-ATPase;

- Analisar alterações causadas pelo tratamento com Hcy, em cultura primária de astrócitos corticais; altera as defesas antioxidantes e na resposta inflamatória;

- Determinar o possível mecanismo de ação da Hcy em astrócitos.

III. METODOLOGIA E RESULTADOS

Os capítulos I, II e III serão apresentados na forma de artigos científicos, sendo que os artigos I e II apresentam o mesmo desenho experimental de HHcy leve aguda com um pré tratamento de vitamina D. E no artigo III foi realizado um modelo experimental de cultura primária de astrócitos de ratos adultos com diferentes concentrações de homocisteína.

Capítulos I e II - Modelo experimental de hiperhomocisteinemia leve aguda com pré tratamento de vitamina D - Fatias

Ratos Wistar machos com 90 dias foram eutanasiados por decapitação, o cérebro foi dissecado e as estruturas desejadas foram removidas e utilizadas para a incubação. Primeiramente as fatias foram incubadas com diferentes doses de vitamina D por 30 min, e após, a Hcy na concentração de 30 μM, foi adicionada na incubação por mais 1 h.

Capítulo III - Modelo experimental de hiperhomocisteinemia leve aguda em cultura primária de astrócitos corticais de ratos adultos

Ratos Wistar machos com 90 dias foram eutanasiados por decapitação e, o córtex cerebral foi retirado assepticamente. Essa estrutura foi dissociada mecanicamente em soluções específicas, centrifugada e após esses procedimentos as células foram ressuspendidas em meio específico para cultivo celular, em seguida colocadas em placas e cultivadas em incubadora. Quando atingida a confluência, a Hcy foi adicionada em diferentes concentrações por 24 h.

Capítulo I

Homocysteine causes changes in the mitochondrial function and redox status in heart slices: Prevention by 1,25-dihydroxyvitamin D3

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Periódico:

Status: Submetido

Abbreviations:

CAT – catalase;

COX - cytrochome c oxidase.

DCFH 2′,7′-Dihydrodichlorofluorescein;

DCFH-DA2′,7′-dihydrodichlorofluorescein diacetate;

DCIP - 2,6-dichloroindophenol;

DMSO –dimethylsulfoxide;

DNPH - 2,4-dinitrophenylhydrazine;

DTNB - 2-nitrobenzoic acid;

GPx - Glutathione peroxidase;

Hcy – homocysteine;

HHcy – hyperhomocysteinemia;

MTG - Mito-Tracker Green FM;

MTR - MitoTracker Red CMXRos;

NADPH - Nicotinamide adenine dinucleotide phosphate;

PI - propidium iodide;

PMS – henazinemethosulfate;

RNS - reactive species nitrogen;

ROS - reactive oxygen species;

SDH - succinate dehydrogenase;

SOD - superoxide dismutase;

TCA - trichloroacetic acid;

Thiobarbituric acid reactive species;

VDR -vitamin D receptor;

Abstract

Because homocysteine (Hcy) is a risk factor for cardiovascular disease and because vitamin D deficiency can contribute to cardiovascular disease, the present study tested the hypothesis that Hcy impairs energy metabolism, mitochondrial function and redox status in heart slices of Wistar rats and that 1,25-dihydroxivitamin D3 (calcitriol) treatment prevents the deleterious effects of Hcy. Heart slices were first pretreated with three different concentrations of calcitriol (50nM, 100nM and 250nM) for 30 min at 37 °C, after which Hcy was added to promote deleterious effects on metabolism. After 1 h of incubation, the samples were washed, homogenized and stored at -80 °C prior to analysis. The results showed that Hcy caused changes in energy metabolism (respiratory chain enzymes), mitochondrial function, and cell viability. Hcy also induced oxidative stress, increasing lipid peroxidation, reactive oxygen species (ROS) generation, and protein damage. An imbalance in antioxidant enzymes was also observed. Calcitriol (50nM) reverted the effect of Hcy on the parameters tested, except for the immunocontent of catalase (CAT). Both treatments (calcitriol and Hcy) did not alter the vitamin D receptor (VDR) immunocontent, which combined with the fact that our *ex vivo* model is acute, suggested that the beneficial effect of calcitriol occurs directly through antioxidative mechanisms and not via gene expression. In this study, we show that Hcy impairs mitochondrial function and induces changes in the redox status in heart slices, which were reverted by calcitriol. These findings suggest that calcitriol may be a preventive/therapeutic strategy for complications caused by Hcy.

1. Introduction

Hcy is a thiol amino acid that is generated from the metabolism of methionine and has been associated with many diseases, including cardiovascular and neurological diseases [1, 2]. In the last decade, clinical studies have shown that mildly elevated plasma levels of Hcy are a risk factor for cardiovascular diseases [3, 4]. Most laboratories report that the normal plasma concentration of Hcy is approximately 4 to 14 µM. Mild hyperhomocysteinemia (HHcy) is considered to occur when Hcy levels reach approximately 15 to 30 µM [5]. This increase in plasma Hcy can be caused by systemic diseases, methylenetetrahydrofolate enzyme reductase deficiency, vitamin deficiency, drug treatment, aging, smoking and alcohol consumption [6-8].

Mild HHcy seems to be related to endothelial injuries and oxidative stress [9, 10], but the underlying mechanisms are poorly understood. Studies have shown that Hcy may contribute to the development and progression of atherosclerosis by inducing endothelial dysfunction, increasing the proliferation of vascular smooth muscle cells and promoting the oxidation of lipoproteins and platelet activation [11]. The mechanism by which HHcy promotes cellular death in heart cells is not completely known [12]. However, as with other diseases of the heart, several studies point to oxidative stress as a key factor in Hcy toxicity, leading to vascular and mitochondrial dysfunction that impair cellular energy metabolism [13].

In humans, ergocalciferol and cholecalciferol are transformed into 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), or calcitriol, which is recognized as the most active form of vitamin D [14, 15]. Studies show inverse associations between calcitriol levels and hypertension [16, 17], congestive heart failure [18, 19],

myocardial infarction [20, 21], and stroke [22]. Calcitriol supplementation at moderate to high doses has also been suggested as a means to reduce the risk of cardiovascular disease [23]. In a recent study, our group showed that calcitriol acts as an antioxidant, preventing the oxidative damage induced by Hcy in rats [24].

In the present study, we hypothesize that Hcy promotes oxidative stress (reactive oxygen species, lipid and protein damage and antioxidant enzyme expression) and impairs mitochondrial function (mass and membrane potential) and bioenergetic parameters (SDH, complex II and COX). We also tested the preventive effect of calcitriol against this model. To test our hypothesis, we used an *ex vivo* model involving the incubation of heart slices from adult rats with Hcy (30 µM) following pretreatment with calcitriol.

2. Material and Methods

2.1. Ethics Statement

All experiments were approved by the local Ethics Commission (CEUA/UFRGS) under protocol number 26073 and followed the National Institutes of Health "Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996).

2.2. Animals and chemicals

Male adult Wistar rats (90 days old, n = 6) were obtained from the Central Animal House of the Department of Biochemistry and maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22 ± 2 °C). These conditions were maintained constantly throughout the experiments. Homocysteine, 1,25-dihydroxyvitamin D_3 and all

other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The reagent 1,25-dihydroxyvitamin D_3 was diluted in 3% ethanol. A control group received the same concentration of ethanol, and no differences were observed in this group across all analyses (data not shown).

2.3. Heart preparation and *ex vivo* incubation with Hcy and calcitriol

The rats were killed by decapitation, and the heart was dissected and cut into slices (0.3 mm) using a McIlwain tissue chopper. The heart slices (60-80 mg) were allowed to stand for 30 min in Dulbecco's buffer at 37 °C. After this period, the slices were washed with buffer, and the medium was changed in the experimental groups. The slices were pre-incubated in 1.0 mL of Dulbecco's buffer (pH 7.4) containing 5.0 mM D-glucose in a metabolic shaker (60 breaths/min) at 37 °C for 30 min. The solutions also contained calcitriol at three different concentrations. After the preincubation, we added Hcy (30 µM) to the medium for 60 min according to the method described in Morrone, de Assis [25]. The slices were separated into five experimental groups: 1) a control group in which the solution contained only Dulbecco's buffer; 2) a group in which Hcy-containing Dulbecco's buffer was used during the pretreatment period (30 min), after which Hcy was added at a concentration of 30 µM for an additional 60 min; 3) a group in which a solution of calcitriol (50 nM) in Dulbecco's buffer was used during the pretreatment period (30 min), after which Hcy was added at a concentration of 30 µM for 60 min; 4) a group in which Calcitriol (100 nM) in Dulbecco's buffer was used in the pretreatment period (30 min), after which Hcy was added at a concentration of 30 µM for 60 min; and 5) a group in which Calcitriol (250 nM) in Dulbecco's buffer was used during the pretreatment period (30 min), after which Hcy was then added at a concentration of 30 µM for 60 min.

After the incubation, the samples were washed, homogenized in buffer, and stored at -80 °C until analysis.

We generated a calcitriol dose-response curve to determine the safest dose to use (data not shown) because some studies have shown that calcitriol can be toxic at high concentrations [26-29].

2.4. Assays for respiratory chain enzyme activities

2.4.1. Succinate dehydrogenase activity

To analyze succinate dehydrogenase (SDH) activity, heart slices were homogenized 1:20 (w/v) in SETH buffer pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base and 50 UI.mL−1 heparin). The homogenates were centrifuged at 800 g for 10 min, and the supernatants were stored at -70 °C until use for the determination of enzyme activities and protein content. SDH activity was determined according to the method of Fischer, Ruitenbeek [30] by measuring the decrease in the absorbance of 2,6-dichloroindophenol (DCIP) at 600 nm, with 700 nm as reference wavelength (ε=19.1 mM−1 cm−1) in the presence of phenazinemethosulfate (PMS). For this test, the samples were thawed and refrozen three times to break the mitochondrial membrane. The reaction solution containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 µM DCIP was pre-incubated with 40–80 µg of protein homogenate at 30 °C for 20 min. Afterwards, 4 mM sodium azide, 7 µM rotenone and 40 µM DCIP were added. The reaction was initiated by the addition of 1 mM PMS and was measured after 5 min. The results were expressed as nmol/min.mg protein.

2.4.2. Complex II activity

Complex II activity was measured by monitoring the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm, with 700 nm as reference wavelength (ε=19.1 mM−1 cm−1), according to Fischer, *et al.* [30]. Immediately prior to testing, the samples were thawed and refrozen three times to break the mitochondrial membrane. The reaction solution containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 µM DCIP was pre-incubated with 40–80 µg of protein homogenate at 30 °C for 20 min. Afterwards, 4 mM sodium azide and 7 µM rotenone were added. The reaction was initiated by the addition of 40 µM DCIP and was measured after 5 min. The results were expressed as nmol/min.mg protein.

2.4.3. Cytochrome c oxidase

The activity of this enzyme was determined according to Rustin, Chretien [31]. The enzymatic activity was measured at 25 °C for 10 min by the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm, with 580 nm as a reference wavelength (ε=19.1 mM−1 cm−1). The reaction solution contained 10 mM potassium phosphate, pH 7.0, 0.6 mM n-dodecyl-β-D-maltoside, and 2–4 µg of protein homogenate, and the reaction was initiated by the addition of 7.0 µg of reduced cytochrome c. The results were expressed in nmol/min.mg protein.

2.5. Flow cytometry

2.5.1. Cell isolation from heart slices

Cardiac myocytes were isolated from heart slices as described by Hilal-Dandan, Kanter [32]. Briefly, slices were first exposed to an enzymatic digestion

buffer (113 mM NaCl; 4.4 mM KCl; 0.6 mM KH_2PO_4; 0.6 mM Na_2HPO_4; 1.2 mM $MgSO_4 \cdot 7H_2O$; 12 mM $NaHCO_3$; 12 mM $KHCO_3$; 20 mM D-Glucose; 10 mM Na+-HEPES, pH 7.2) supplemented with 0.08% collagenase Type II, 0.1% trypsin, 0.1% BSA, 10 mM 2,3-butanedione monoxime, and 25 µM Ca^{2+}. Smaller tissue pieces were placed in tubes containing Ca^{2+}-free medium, and cells were released by gentle trituration through a serologic pipette just before filtration through a 200-µM nylon mesh. Filtered cells were washed by centrifugation at 3000 rpm for 5 min at 4 °C, and the resulting pellets were assayed.

2.5.2. Mitochondrial mass and membrane potential measurements

MitoTracker Red CMXRos (MTR) and Mito-Tracker Green FM (MTG) dyes were employed to assess mitochondrial function and morphology/mass, respectively. Briefly, MitoTracker probes passively diffuse across the plasma membrane to accumulate in mitochondria. MTR is a chloromethyl X rosamine-based dye whose red fluorescence is increased by the mitochondrial membrane potential, whereas MTG is a carbocyanine-based dye whose green fluorescence is increased by mitochondrial mass independent of mitochondrial membrane potential [33]. Therefore, the relationship between MTR and MTG staining indicates the rate of mitochondrial function.

MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to a 1-mM stock concentration. Dissociated cells were stained with 100 nM MTR and 100 nM MTG for 45 min at 37 °C in a water bath in the dark, according to [33, 34] with some modifications. Immediately after staining, cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA). A total of 10,000 events were acquired in the FL-1 (green fluorescence) and FL3 (red

fluorescence) channels. All data analyses were performed with FCS Express 4 software (De Novo, Software, Ontario, Canada)

2.5.3. Cell Viability

Plasma membrane integrity was assessed by determining the ability of cells to exclude PI. Briefly, dissociated cells were suspended in PBS containing 10 µg/ml propidium iodide (PI) for 30 min at room temperature in the dark. Then, cells were washed by centrifugation at 3000 rpm for 5 min at 4 °C to remove free PI [35]. After staining, cell suspensions were analyzed by flow cytometry (FACSCalibur). A total of 10,000 events were acquired in the FL3 (red fluorescence) channel. All data analyses were performed with FCS Express 4 software (De Novo, Software, Ontario, Canada)

2.6. Oxidative stress parameters

2.6.1. Thiobarbituric acid reactive species (TBARS)

To assess the extent of lipoperoxidation, we detected TBARS formation through a heated and acidic reaction. This approach is widely adopted to measure lipid redox states, as previously described [36]. Homogenized heart samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid and were then heated for 25 min in a boiling water bath. The level of TBARS was determined by measuring the absorbance of each sample at 532 nm. The concentration of TBARS in the samples was then determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. The results are expressed as nmol of TBARS/mg protein.

2.6.2. Measurement of protein carbonyl content

Oxidative damage to proteins was measured by quantifying the number of carbonyl groups using a 2,4-dinitrophenylhydrazine (DNPH) reaction. Proteins were precipitated from homogenized heart samples by the addition of 20% TCA and resuspended in 10 mM DNPH, and the absorbance of the resulting solution at 370 nm was recorded [37]. The results are expressed as nmol carbonyl/mg protein.

2.6.3. 2′,7′-Dihydrodichlorofluorescein oxidation

Reactive oxygen/nitrogen species production was measured following the method of [38], which is based on DCFH oxidation. 2′,7′-dihydrodichlorofluorescein diacetate (DCFH-DA) is cleaved by cellular esterases, and the resulting DCFH is eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) present in samples. Briefly, DCFH-DA was incubated for 30 min at 37 °C with an aliquot of heart homogenate. Fluorescence was measured at 488 nm excitation and 525 nm emission. A calibration curve was generated with standard DCF, and reactive species levels were calculated as nmol of DCF/mg protein.

2.6.4. Total protein thiol content

After incubation, tissue samples were analyzed for protein thiol content, which was used to estimate oxidative alterations in proteins. As previously described by Ellman [39], an aliquot of sample was diluted in 0.1% SDS, after which 0.01 M 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in ethanol was added. An intense yellow color developed and was read in a spectrophotometer at 412 nm after 60 min.

2.6.5. Superoxide dismutase activity assay

To determine superoxide dismutase (EC 1.15.1.1) (SOD) activity, we used homogenized heart samples (40 µg of protein) plus 150 µL of 50 mM glycine buffer, pH 10.2, and 10 µl of 10 µM Catalase (CAT) added on the microplate. As a control, 180 µl of 50 mM glycine buffer, pH 10.2, and 10 µl of 10 µM CAT were added to another well. The reaction was initiated by adding 10 µl of 60 mM epinephrine. The absorbance at 480 nm was measured at time zero and again after 10 min at 32 °C. A unit of SOD activity was defined as the amount of enzyme required to decrease the formation of superoxide by 50%. The specific activity was expressed as units/mg protein [40].

2.6.6. Catalase activity assay

Catalase (EC 1.11.1.6) (CAT) activity was measured as previously described [41]. Homogenized heart samples (60 µg of protein) were mixed with 150 µl of phosphate buffer (125 mM, pH 7.4). The reaction was initiated by adding 10 µl of H_2O_2 (0.5 mM). A blank was prepared with 190 µl of phosphate buffer and 10 µl of H_2O_2 (0.5 mM). The rate of decrease in optical density was measured at 240 nm after 1 min with respect to the blank. A unit of CAT activity was defined as the amount of enzyme that decomposed 1 mM H_2O_2 per minute at 37 °C. The specific activity was expressed as units/mg protein.

2.6.7. Glutathione peroxidase activity assay

Glutathione peroxidase (EC 1.11.1.9) (GPx) activity was measured according to the method described by [42] using *tert*-butyl hydroperoxide as a substrate. The disappearance of nicotinamide adenine dinucleotide phosphate (NADPH) was monitored at spectrophotometrically at 340 nm in medium

containing 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 µmol of NADPH consumed per minute, and the specific activity is represented as units/mg protein.

2.7. Western blotting

Proteins (20-30 µg) were separated via 10% (w/v) SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were incubated in TBS-T (20 mmol/L Tris-HCl, pH 7.5, 137 mmol/L NaCl, 0.05% (v/v), and Tween 20) containing 5% (w/v) bovine serum albumin for 1 h at room temperature. The membranes were then incubated for overnight with the appropriate primary antibody against catalase (Cell Signaling®, dilution 1:1000), superoxide dismutase 2 (Cell Signaling®, dilution 1:5000), vitamin D receptor (Abcam®, dilution 1:1000) and β-actin (Sigma Aldrich®, dilution 1:2000), rinsed with TBS-T, and exposed to horseradish peroxidase-linked anti-IgG antibodies for 2 h at room temperature. Chemiluminescent bands were detected using an ImageQuant LAS4000 GE Healthcare®, and densitometry analyses were performed using Image-J® software. The results were expressed as percentage of control [43].

2.8. Statistical analyses

Data are expressed as the mean ± S.E.M. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0, Chicago, IL, USA) software. Differences among groups were analyzed using one-way ANOVA and Tukey's *post hoc* test with the following levels of significance: *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

The Z-score was calculated using the following formula:

$$Z = \frac{x - \mu}{\sigma}$$

Where x is the sample value (raw score), μ is the population mean and σ is the standard deviation of the population. The absolute value of Z represents the distance between the raw score and the population mean in units of standard deviation [44].

3. Results

Figure 1 shows that Hcy decreased the activities of SDH (1A, $P<0.01$), Complex II (1B, $P<0.05$) and cytochrome c oxidase (1C, $P<0.05$). Post hoc analysis showed that only pretreatment with 50 nM calcitriol was able prevent the effect of Hcy on the activities of these enzymes.

Mitochondrial function and cell viability were also analyzed. The reduction in the number of MTG/MTR co-labeled cells, indicated by a downward shift towards the MTG-positive gate, revealed that Hcy impaired mitochondrial function, whereas calcitriol (50 nM mainly) prevent this effect (2A, $P>0.05$). The mean of the relative fluorescence of MTG and MTR (2B and 2C, $P<0.05$) revealed that Hcy promotes a significant increase in mitochondrial mass but did not affect the mitochondrial membrane potential. Additionally, the ratio of MTG and MTR fluorescence (2D, $P>0.05$) suggests an increase in swollen and nonfunctional mitochondria that was prevented by 50 nM calcitriol. Accordingly, cell viability was decreased by Hcy (2E and 2F, $P<0.05$, $P<0.01$ and $P<0.001$), while calcitriol (50 nM mainly) prevented this effect.

Because mitochondrial dysfunction induces oxidative stress [13], we investigated if Hcy modulates redox status in an *ex vivo* model and if calcitriol

acts as an antioxidant to prevent the alterations induced by Hcy. Figure 3 shows that Hcy increased lipid peroxidation (3A, $P<0.01$), protein carbonyl content (3B, $P<0.01$), and ROS (3C, $P<0.05$) and decreased thiol content in rat heart slices (3D, $P<0.05$). Calcitriol (50 nM) was able to prevent the deleterious effects of Hcy, although 100 nM calcitriol prevented only the effect of Hcy on TBARS and protein carbonyl content (Figure 3A and 3B, $P<0.05$).

Regarding antioxidant enzymes, Figure 4 shows that Hcy significantly decreased the activities of SOD (4A, $P<0.05$) and CAT (4C, $P<0.05$), while GPx activity was increased (4B, $P<0.05$). Preincubation with 50 nM calcitriol reversed the effects of Hcy ($P<0.05$). Figure 5 shows that Hcy decreased SOD and CAT immunocontent (5A, $P<0.05$ and 5B, $P<0.01$) but that calcitriol was not able to rescue the SOD and CAT immunocontent at any concentration tested.

We also tested the effect of Hcy on vitamin D receptor immunocontent. The results showed that this amino acid did not alter vitamin D receptor levels (Figure 5C).

4. Discussion

Several studies have demonstrated a correlation between Hcy levels and the risk for cardiovascular diseases [12, 45]. However, despite many advances in our understanding of this condition, few effective strategies for cardioprotection are currently available. In the present study, we evaluated the effect of Hcy on some parameters of energy metabolism, mitochondrial function and redox status, as well as on the immunocontent of antioxidant enzymes and vitamin D receptor in heart slices of rat.

To see how Hcy behaves in relation to the respiratory chain in heart slices, we evaluated the activities of SDH, complex II and cytochrome c oxidase. Our results showed that Hcy reduced the activities of SDH, complex II and cytochrome c oxidase, suggesting that low concentrations of this amino acid, when added to incubation medium (ex vivo study), may compromise the function of the respiratory chain in heart slices and that calcitriol was able to reverse these effects. Mitochondrial oxidative phosphorylation is known to generate free radicals, and electron transport chain complexes are vulnerable to free radicals [46, 47]. Cytochrome c oxidase is a marker of oxidative phosphorylation; in the respiratory chain, it catalyzes a rate-limiting step, transferring electrons from cytochrome C to molecular oxygen [48]. Therefore, the inhibition of this enzyme may lead to incomplete reduction of oxygen and consequently to an increase in the formation of free radicals [49].

Hcy caused an increase in nonfunctional and swollen mitochondria, suggesting possible alterations in organelle membrane permeability to favor the entry of water and other substances. The transfer of electrons through the four protein complexes are accompanied by the pumping of protons outward from the array to the intermembrane space, creating an electrochemical gradient, which is referred to as the membrane potential. This potential can be considered a key indicator in mitochondrial function and metabolic activity [50, 51]. The changes in mitochondrial function caused by Hcy can lead to substantial effects on cell energy homeostasis. On the other hand, we also observed that calcitriol was able to reverse such effects.

Because mitochondrial dysfunction can lead to free radical formation and/or oxidative stress induction, we also investigated the effect of Hcy on some

parameters of redox status in heart slices. We verified that this amino acid promoted increased lipid peroxidation and reactive oxygen species production, as well as protein damage, indicated by increased carbonyl content and reduced thiol content [52-54]. Regarding antioxidant enzymes, Hcy caused a decrease in the activities of SOD, CAT and GPX, as well as a decrease in SOD and CAT immunocontent. These results suggest that Hcy provokes an antioxidant imbalance in rat heart slices, suggesting that the antioxidant system was not very effective and that there was an increase in the production of superoxide and hydrogen peroxide. These data are in agreement with previous studies, which show that during the oxidation of Hcy, reactive species such as O_2- and $OH-$, which have detrimental effects on most cellular components, can be generated [55]. In this context, the main sources of reactive oxygen species in cardiac infarction are thought to be the mitochondrial electron transport chain of NADPH oxidase and/or auto-oxidation of many substances, such as Hcy [56]. This amino acid acts as a strong oxidizing agent for the production of reactive species such as H_2O_2 and O_2 during autoxidation [57]. The increase in reactive oxygen species caused by Hcy can lead to endothelial dysfunction and damage to the vessel wall, followed by thrombus formation via platelet activation [9, 45]. We observed that calcitriol can prevent the effect of Hcy on reactive oxygen species production, on lipid and protein damage, and on decreased antioxidant enzymes activities, but it did not protect against the reduction of antioxidant enzymes immunocontent.

The participation of the vitamin D receptor (VDR) in mediating the cardioprotective effects of calcitriol is still controversial [58]. Likewise, we observed a cardio protective effect of calcitriol, though the intracellular

modulation of this substance seems not be through the VDR, as we did not see any difference in the level of this protein following pretreatment with calcitriol. Interestingly, we did not see any effect of calcitriol on antioxidant enzyme immunocontent (SOD and CAT), but we observed that 1,25-dihydroxyvitamin D3 prevented the decay of the activities of these enzymes. Considering that ours and other studies used an acute experimental model [59, 60], these results indicate that the beneficial actions of calcitriol probably involve improved antioxidant activity to disrupt pro-oxidative signaling, as opposed to gene expression via VDR pathway.

5. Conclusion

Analyzing all the results (Figure 6), we suggest that Hcy causes changes in the mitochondrial function and redox status in heart slices, which may be related to cardiovascular complications due to Hcy tissue levels, and that 50 nM calcitriol was able to reverse these changes and protect against most Hcy effects. Notably, ours is the first study to analyze the effect of Hcy with calcitriol in an acute model of heart slices. More studies are needed to understand the mechanisms by which calcitriol exerts cardioprotective effects and whether calcitriol can be considered a new therapeutic target for preventing or retarding cardiovascular dysfunction in individuals with mild HHcy.

6. Acknowledgments

financial or other conflict of interest relevant to the subject of this article.

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Figure Legends.

Figure 1. Cardioprotective effect of calcitriol pretreatment against Hcy-induced cellular dysfunction in rat heart slices on the activities of respiratory-chain enzymes: succinate dehydrogenase-SDH (A), complex II (B) and cytochrome c oxidase (C). The results are expressed in nmol/min/mg of protein as the mean ± S.E.M. (n = 6 per group). *$P<0.05$ versus the control according to one-way ANOVA, followed Tukey's post-hoc test.

Figure 2. Cardioprotective effect of calcitriol pretreatment against Hcy-induced heart cell dysfunction: Dot plot from flow cytometry indicating the shift to the MTG-positive gate (loss of function) (A), mitochondrial mass (B), and mitochondrial membrane potential (C). Ratio between mitochondrial mass and activity (D) and cell viability (Dot plot from flow cytometry and graphic) (E). All results are expressed as the mean ± S.E.M of the number of cells. (n = 6 per group). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ versus the control according to one-way ANOVA, followed Tukey's post-hoc test.

Figure 3. Cardioprotective effect of calcitriol pretreatment against Hcy-induced cellular dysfunction in rat heart slices on oxidative damage on TBARS (A), protein carbonyl content (B), DCFH (C), and SH (D). The results are expressed as the mean ± S.E.M in nmol/mg of protein. (n = 6 per group). *$P<0.05$ versus the control according to one-way ANOVA, followed Tukey's post-hoc test.
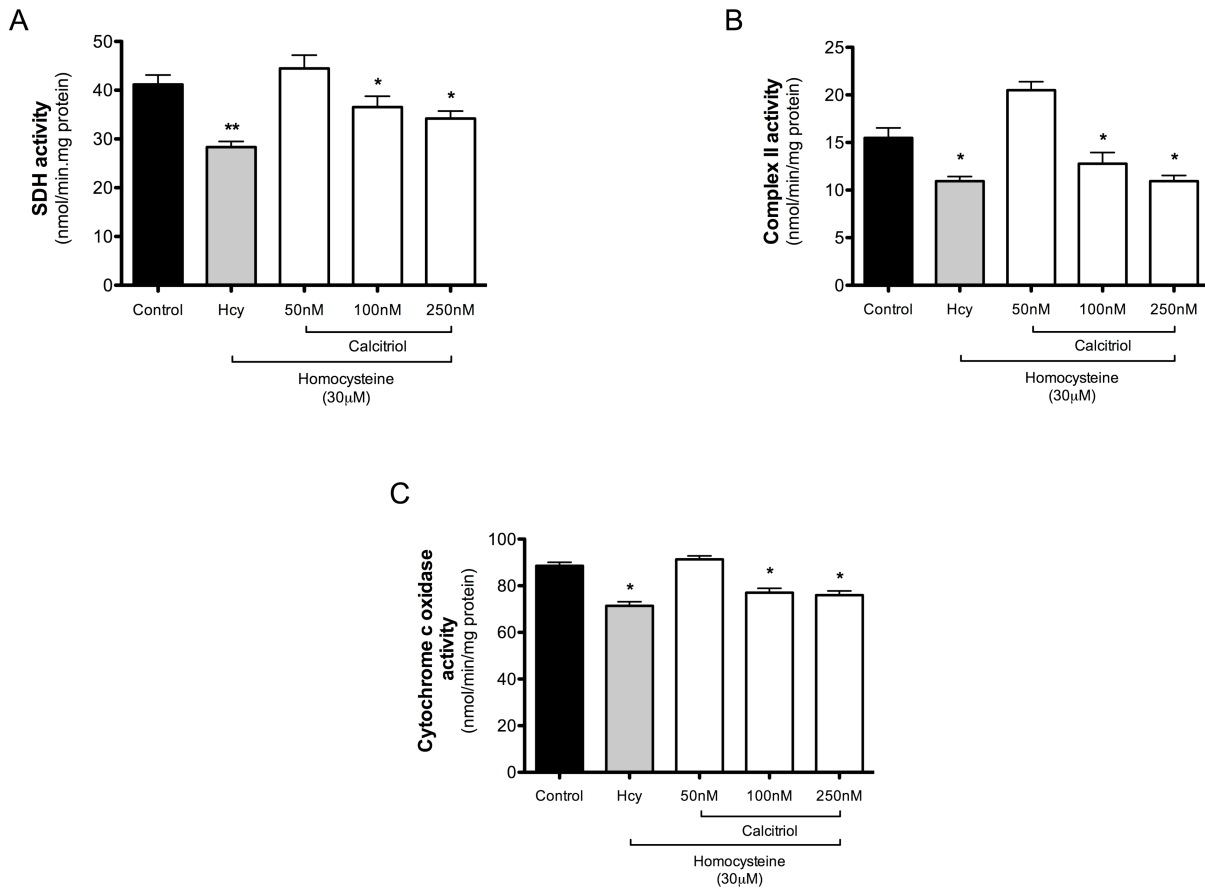
Figure 4. Cardioprotective effect of calcitriol pretreatment against Hcy-induced cellular dysfunction in rat heart slices on superoxide dismutase (A), glutathione peroxidase (B) and catalase (C). Glutathione peroxidase is expressed as % of control, and superoxide dismutase and catalase are expressed in U/mg of

protein. (mean ± S.E.M; n = 6 per group). *P<0.05 versus the control according to one-way ANOVA, followed Tukey's post-hoc test.
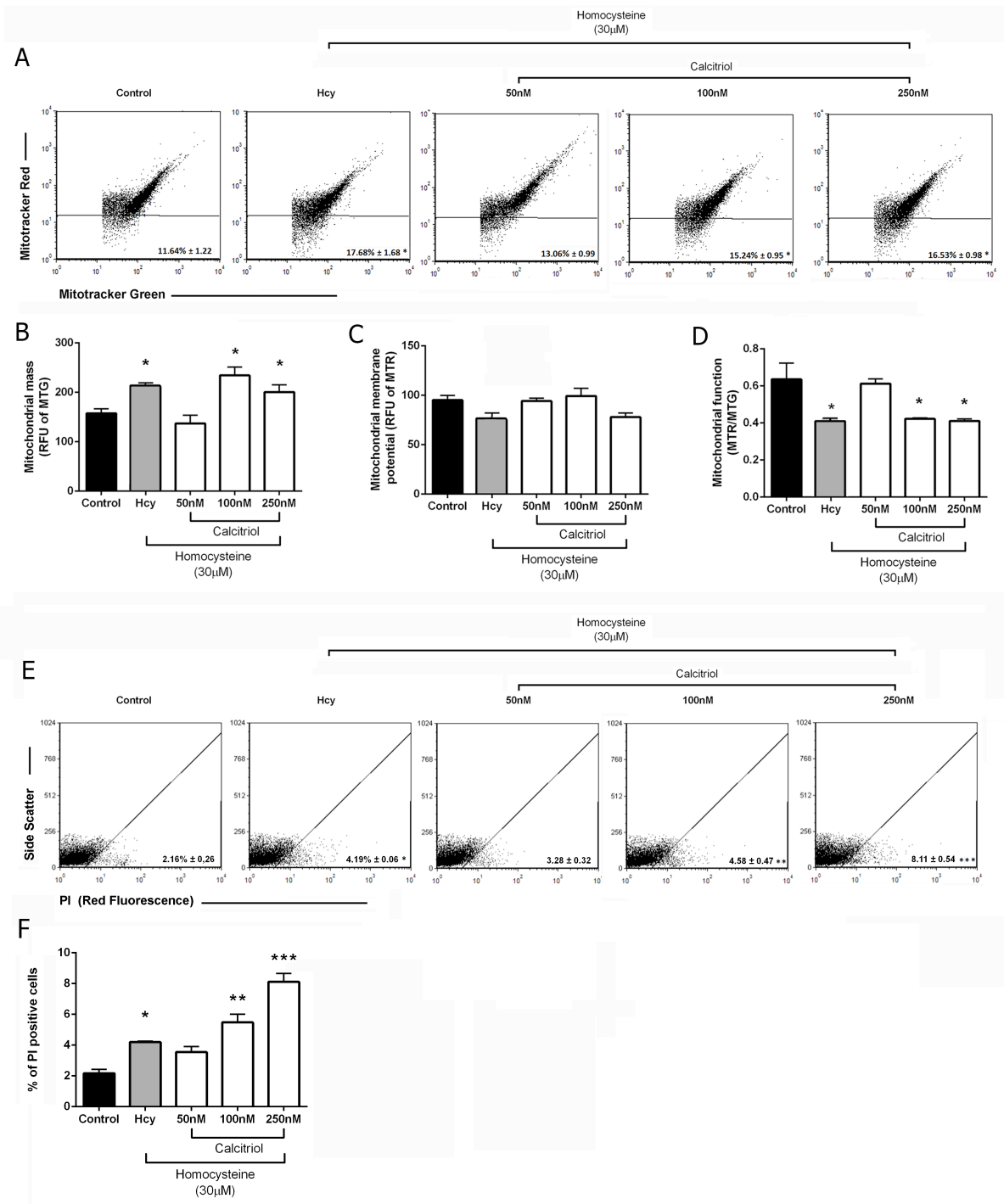
Figure 5. Cardioprotective effect of pretreatment against Hcy-induced cellular dysfunction in rat heart slices on SOD (A), CAT (B) and VDR immunocontent (D). The results are expressed as the mean ± S.E.M in fold control/β-actin. (n = 6 per group). *P<0.05 versus the control according to one-way ANOVA, followed Tukey's post-hoc test.

Figure 6. Summary of the cardioprotective effect of 50 nM calcitriol pretreatment against Hcy-induced cellular dysfunction in rat heart slices. The results were analyzed as Z-score values. Z is negative when the sample value is below the mean and positive when above the mean. We chose 50 nM calcitriol because this was the only concentration that prevented the deleterious effect of Hcy on all parameters analyzed. Each square represents one rat (n=5 per group).
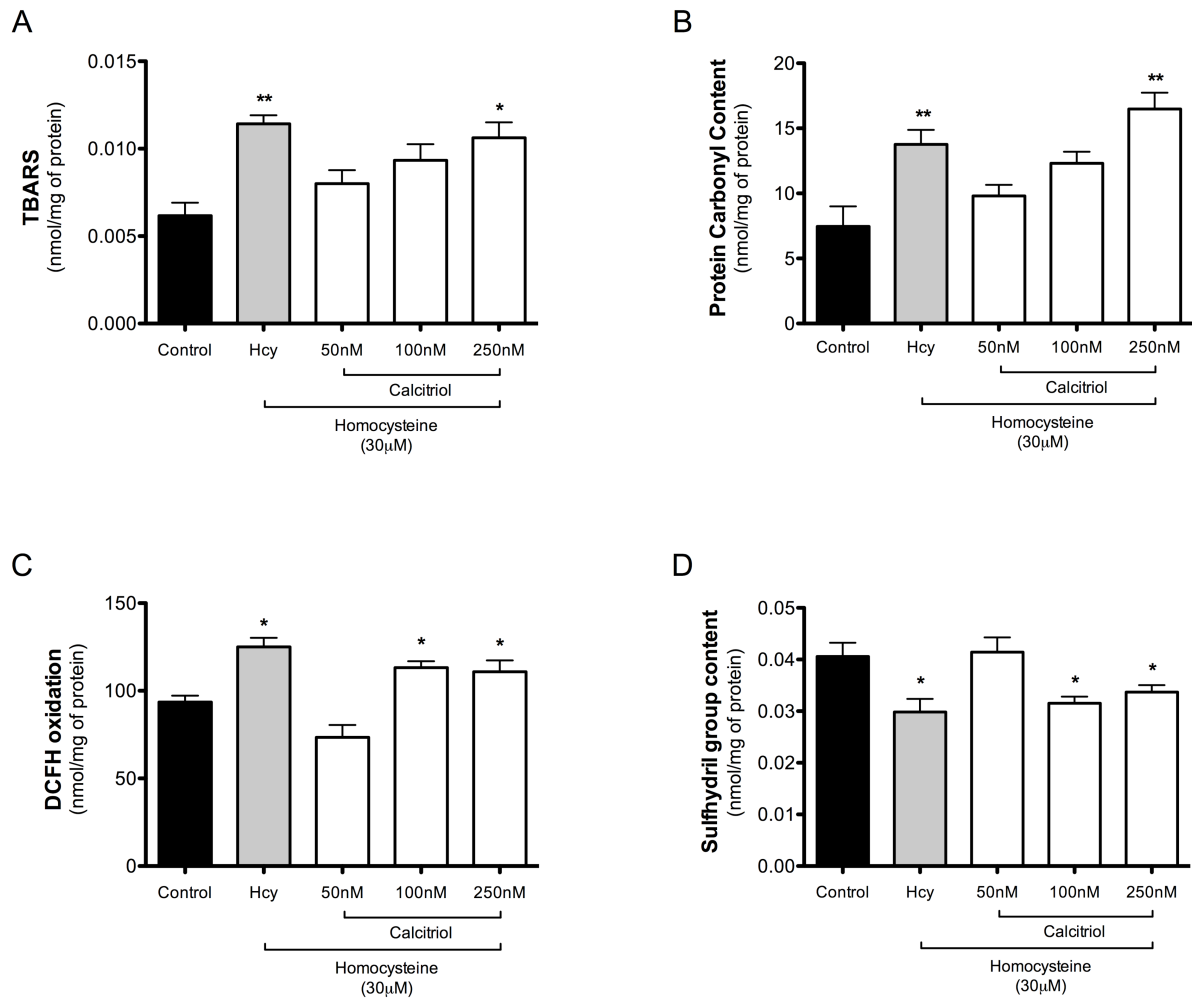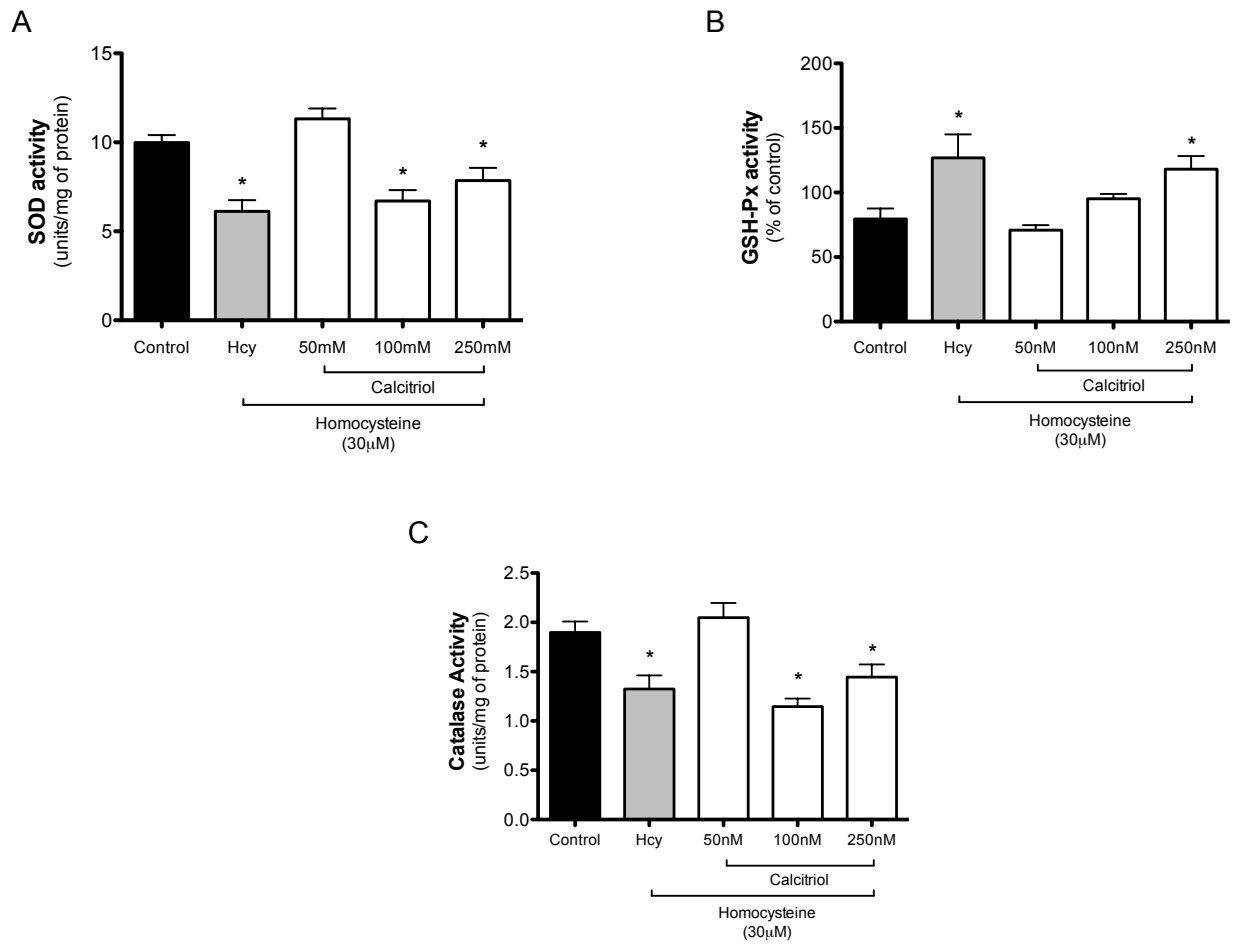
Figure 1.

Figure 2.

Figure 3.

A

TBARS
(nmol/mg of protein)

B

Protein Carbonyl Content
(nmol/mg of protein)

C

DCFH oxidation
(nmol/mg of protein)

D

Sulfhydril group content
(nmol/mg of protein)

Figure 4.

Figure 5.

Figure 6.

A

B

Color Key

-1   0   +1

Row Z-Score

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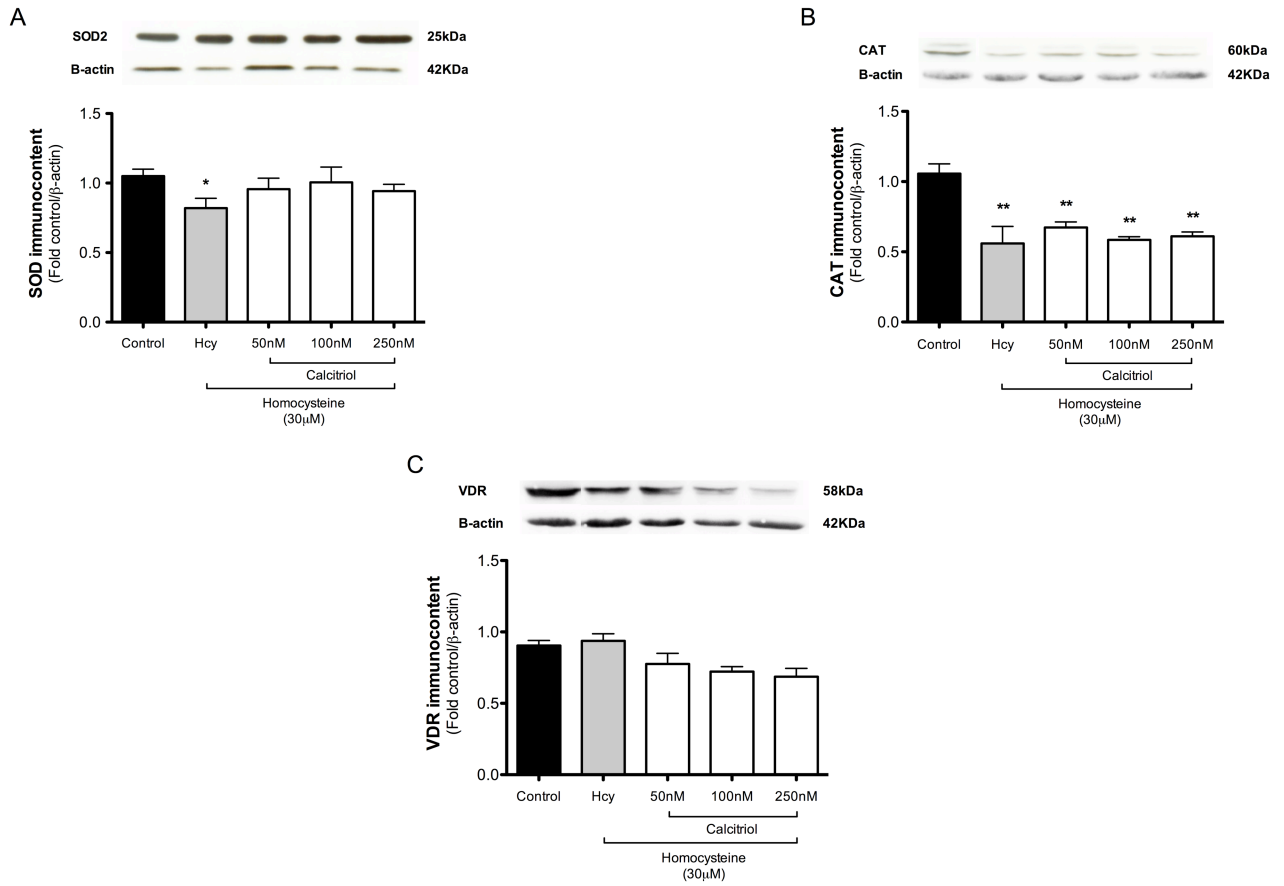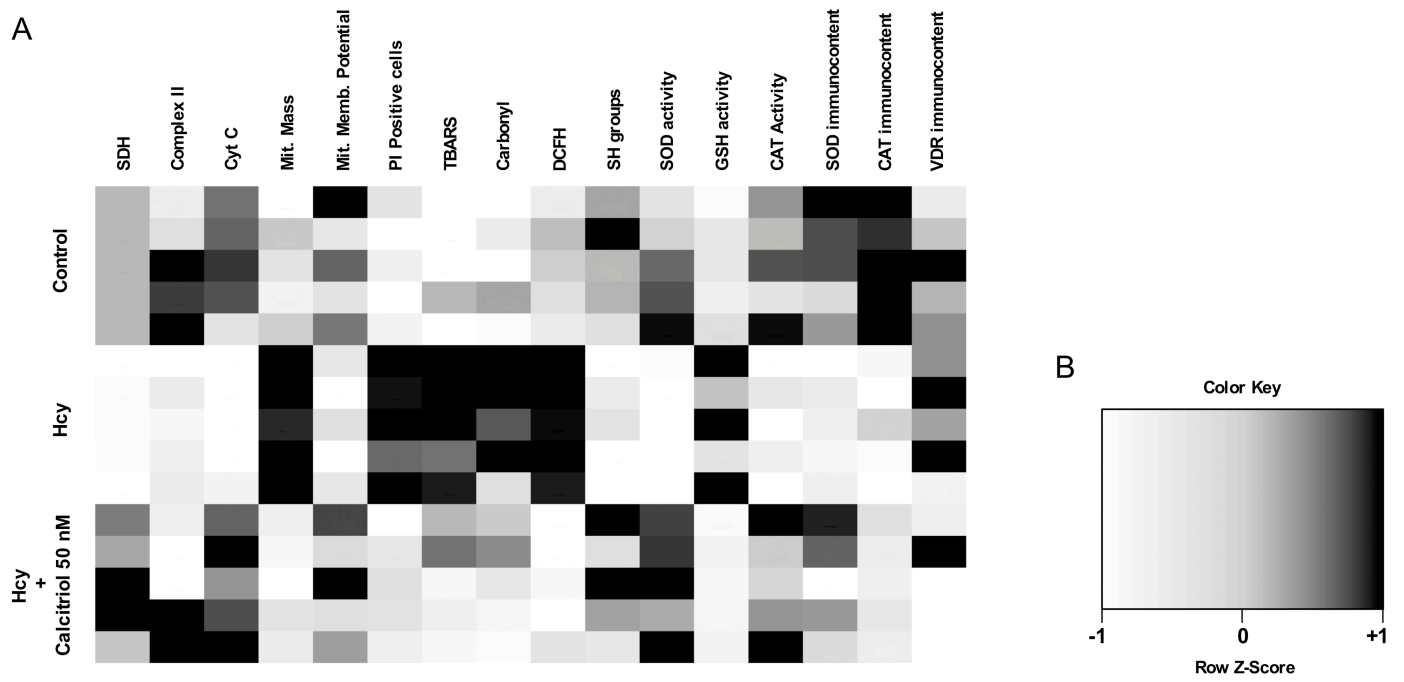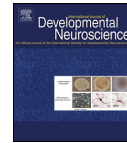journal homepage: www.elsevier.com/locate/ijdevneu

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1,25-Dihydroxyvitamin D3 exerts neuroprotective effects in an *ex vivo* model of mild hyperhomocysteinemia

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ABSTRACT

Elevated plasma homocysteine (Hcy) levels have been detected in patients with various neurodegenerative conditions. Studies of brain tissue have revealed that hyperhomocysteinemia may impair energy metabolism, resulting in neuronal damage. In addition, new evidence has indicated that vitamin D plays crucial roles in brain development, brain metabolism and neuroprotection. The aim of this study was to investigate the neuroprotective effects of 1,25-dihydroxivitamin D3 (calcitriol) in cerebral cortex slices that were incubated with a mild concentration of Hcy. Cerebral cortex slices from adult rats were first pretreated for 30 min with one of three different concentrations of calcitriol (50 nM, 100 nM and 250 nM), followed by Hcy for 1 h to promote cellular dysfunction. Hcy caused changes in bioenergetics parameters (e.g., respiratory chain enzymes) and mitochondrial functions by inducing changes in mitochondrial mass and swelling. Here, we used flow cytometry to analyze neurons that were double-labelled with Propidium Iodide (PI) and found that Hcy induced an increase in NeuN$^+$/PI cells but did not affect GFAP$^+$/Pi cells. Hcy also induced oxidative stress by increasing reactive oxygen species generation, lipid peroxidation and protein damage and reducing the activity of antioxidant enzymes (e.g., SOD, CAT and GPx). Calcitriol (50 nM) prevented these alterations by increasing the level of the vitamin D receptor. Our findings suggest that using calcitriol may be a therapeutic strategy for treating the cerebral complications caused by Hcy.

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1. Introduction

Homocysteine (Hcy) is a sulfur amino acid that is formed during the metabolism of dietary methionine (Stipanuk, 2004). The elevation of Hcy levels in the blood plays an important role in the aetiology of various neurological diseases, such as Alzheimer's disease and Parkinson's disease (Baydas et al., 2003; Cankurtaran et al., 2013; Smeyne and Smeyne, 2013), epilepsy (Herrmann and Obeid, 2011; Sachdev, 2004), stroke (Obeid and Herrmann, 2006), neu-

ropsychiatric disorders (Bottiglieri, 2005; Diaz-Arrastia, 2000), and inborn errors of metabolism (Mudd et al., 2001). High levels of Hcy cause oxidative stress in neurons (Cankurtaran et al., 2013; Tjiattas et al., 2004), alter energy metabolism (Streck et al., 2003a), reduce Ca^{2+} influx, and induce apoptosis by activating cation channels (Ovey and Naziroglu, 2015; Tjiattas et al., 2004). The neurotoxicity of Hcy has been known for decades, and several studies have demonstrated the cytotoxicity of Hcy in both *in vitro* (Kim and Pae, 1996; Parsons et al., 1998) and in vivo experimental models (Scherer et al., 2013). Reports indicate that Hcy is considered an important risk factor for cognitive dysfunction (Miller, 2003) and some diseases that affect the central nervous system (CNS) (Kuhn et al., 1998; White et al., 2001).

The pathological pathway relating Hcy to cellular dysfunction has not been fully defined, but oxidative stress seems to be an important contributing factor. In previous studies, our group showed that Hcy increases the levels of reactive oxygen species (ROS) (Scherer et al., 2011b) and that self-Hcy oxidation generates reactive species, significantly impair-

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Fig. 1. The effect of pre-treatment with calcitriol on Hcy-induced changes in brain energy metabolism in rat cerebral cortex slices : SDH (A), COX (B), Na+, K+-ATPase (C), mitochondrial mass (D) and mitochondrial membrane potential (E). The results are expressed in nmol/min/mg of protein (A, B and C), as a% of the control (D and E), and as the mean ± S.E.M. (n= 6 per group). *P< 0.05 compared to the groups without the symbol and # P> 0.05 similar to all groups, as evaluated using two-way ANOVA followed Tukey's post-hoc test.
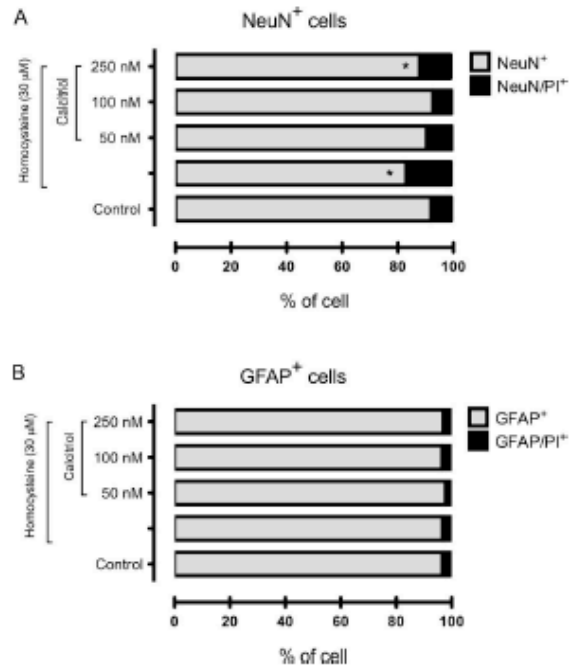
Fig. 2. The effect of pre-treatment with calcitriol before incubation with Hcy in rat cerebral cortex slices on neuronal (A) and astrocytic cell death (B). * indicates differences between NeuN+/NeuN PI+ compared to the other groups (P< 0.05), evaluated using two-way ANOVA followed Tukey's post-hoc test.
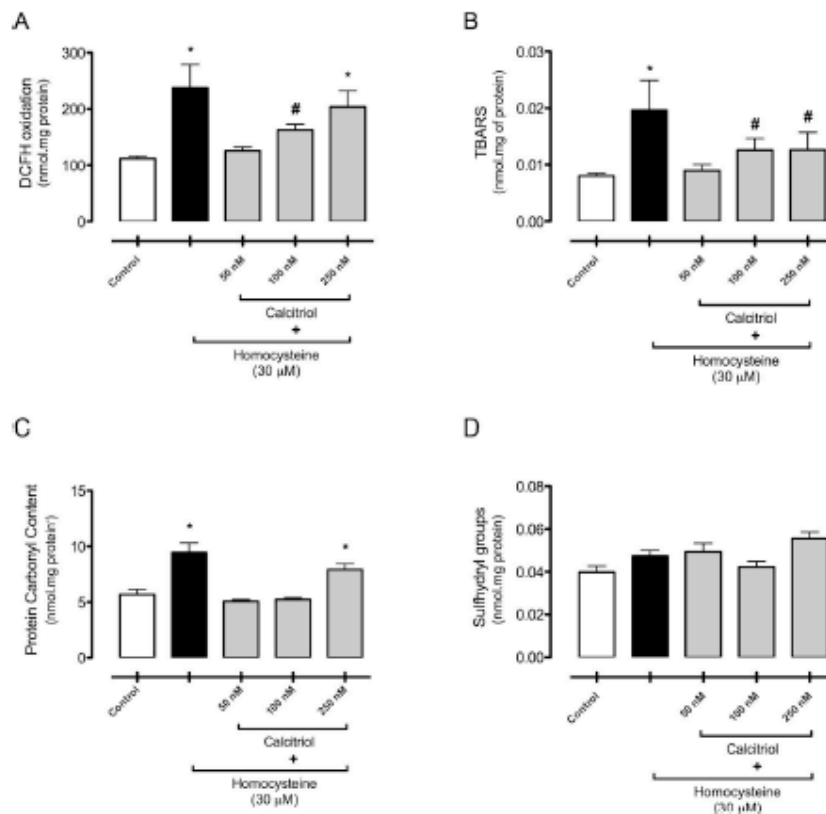
Fig. 3. The effect of pre-treatment with calcitriol against incubation with Hcy in rat cerebral cortex slices on oxidative damage. The effects on TBARS (A), DCFH (B), protein carbonyl content (C), and SH (D) are shown. Results are expressed in nmol/mg of protein and as the mean ± S.E.M. ($n = 6$ per group). * indicates $P < 0.05$ compared to the groups without the symbol and # indicates $P > 0.05$ similarity to all groups, as evaluated using two-way ANOVA followed by Tukey's *post-hoc* test.

ing cellular components (Nishio and Watanabe, 1997; Tyagi, 1998; Welch et al., 1998).

Interactions between neurons is fundamental to brain energy metabolism. Na^+, K^+-ATPase activity is highly related to pathological conditions that affect the CNS because the ionic control of neuronal activity and neurotransmitters (Jorgensen et al., 2003) consumes approximately 40–50% of the ATP generated in the brain (Erecinska and Silver, 1994). On the other hand, the brain is vulnerable to elevated plasma levels of Hcy because this amino acid is transported through the membrane, leading to its intracellular accumulation (Grieve et al., 1992). It has been demonstrated that hyperhomocysteinemia significantly reduces the activity and immunocontent of Na^+, K^+-ATPase and impairs energy metabolism in adult rat brains (Scherer et al., 2013; Streck et al., 2003b).

Recent studies have demonstrated a neuroprotective role for 1,25-dihydroxyvitamin D3 (calcitriol, the active form of vitamin D3) in experimental models of neurodegenerative diseases (Jang et al., 2014; Jang et al., 2015; Newmark and Newmark, 2007). Others studies have shown that calcitriol improved energy metabolism mainly by affecting Na^+, K^+-ATPase activity (Cross and Peterlik, 1983; Elstein and Silver, 1986). The specific mechanisms that mediate this effect are unclear; however, calcitriol acts in many pathways, including the following: i) antioxidant pathways, ii) neuronal calcium regulation, iii) immunomodulation and iv) the glutamatergic system (Shinpo et al., 2000; Taniura et al., 2006; Wang et al., 2001). In another study, Kriebitzsch et al. (2011), showed that in murine and human cells, treatment with calcitriol

induced the transcription of many genes, including cystathionine β-synthase (CBS), which is the first enzyme in the transsulfuration pathway, leading to an increase in CBS activity and a reduction in Hcy levels.

Based on these findings, the hypothesis for this study was that Hcy promotes neural cell dysfunction, leading to impairments in brain energy metabolism, mitochondrial function and redox status. We sought to determine whether calcitriol pre-treatment would be able to prevent these deleterious effects of Hcy. To test our hypothesis, we used an *ex vivo* model involving the incubation of cerebral cortex slices from adult rats with Hcy following pre-treatment with calcitriol.

2. Material and methods

2.1. Ethics statement

All experiments were approved by the local Ethics Commission—CEUA/UFRGS under the number 26073 and followed the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996).

2.2. Animals and chemicals

Male adult Wistar rats (90 days old, $n - 6$) were obtained from the Central Animal House of the Department of Biochemistry and maintained under a standard dark–light cycle (lights on between

7:00 a.m. and 7:00 p.m.) at room temperature ($22 \pm 2\,°C$). These conditions were maintained constantly throughout the experiments. Homocysteine, 1,25-dihydroxyvitamin D_3 and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). The reagent 1,25-dihydroxyvitamin D_3 was diluted in 3% ethanol. A control group was formed using the same concentration of ethanol, and no differences were observed in this group across all analyses (data not shown).

2.3. Cerebral cortex preparation and ex-vivo incubation with Hcy and calcitriol

The rats were killed by decapitation, and the cerebral cortex was dissected and cut into slices (0.3 mm) using a McIlwain tissue chopper. The cerebral cortex slices (100–120 mg) were allowed to stand for 30 min in Dulbecco's buffer at 37 °C. After this period, the slices were washed with buffer, and the medium was changed in the experimental groups. The slices were pre-incubated in 1.0 mL of Dulbecco's buffer (pH 7.4) containing 5.0 mM D-glucose in a Dubnoff metabolic shaker (60 breaths/min) at 37 °C for 30 min. The solutions also contained calcitriol at three different concentrations. After the pre-incubation, we added Hcy (30 μM) to the medium for 60 min according to the method described in Morrone et al. (2013). The slices were separated into five experimental groups: 1) a control group in which the solution contained only Dulbecco's buffer; 2) a group in which Hcy-containing Dulbecco's buffer was used during the pre-treatment period (30 min), and then Hcy was added at a concentration of 30 μM for an additional 60 min; 3) a group in which a solution of Calcitriol (50 nM)-containing Dulbecco's buffer was used during the pre-treatment period (30 min), and then Hcy was added at a concentration of 30 μM for 60 min; 4) a group in which Calcitriol (100 nM)–containing Dulbecco's buffer was used in the pre-treatment period (30 min), and then Hcy was added at a concentration of 30 μM for 60 min; 5) a group in which Calcitriol (250 nM)–containing Dulbecco's buffer was used during the pre-treatment period (30 min) and Hcy was then added at a concentration of 30 μM for 60 min. After the incubation, the samples were washed, homogenized in a buffer, and stored in a freezer at −80 °C until analysis.

We performed a calcitriol dose-response curve to determine the safest dose to use (data not shown) because some studies have shown that calcitriol can be toxic at high concentrations. We showed that 250 nM of calcitriol induced a significant decrease in cell viability. Several studies in the literature have shown that humans and rodents can tolerate plasma 25(OH)D_3 concentrations up to 500 nmol/L with no toxicity (Jang et al., 2014, 2015; Tukaj et al., 2010; Vieth, 2006).

2.4. Assays to determine respiratory chain enzyme activity

2.4.1. Analysis of Na$^+$, K$^+$-ATPase activity

A reaction mixture was prepared for Na$^+$, K$^+$-ATPase activity assays. It contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl (pH 7.4) in a final volume of 200 μl. After 10 min of pre-incubation at 37 °C, the reaction was begun by adding ATP at a final concentration of 3.0 mM, and the slices were incubated for 20 min. Control experiments were performed under the same conditions but using 1.0 mM ouabain. Na$^+$, K$^+$-ATPase activity was calculated as the difference between the two assays according to them methods described in Wyse et al. (2000). Released inorganic phosphate (Pi) was measured using the methods described in Chan et al. (1986), and enzyme-specific activity was expressed as nmol Pi released per min per mg of protein.

2.4.2. Succinate dehydrogenase activity

To analyse succinate dehydrogenase (SDH) activity, cerebral cortex slices were homogenized 1:20 (w/v) in SETH buffer, pH 7.4 (sucrose 250 mM, 2 mM EDTA, 1 mM Trizma base and 50UI mL^{-1} of heparin). The homogenates were centrifuged at $800 \times g$ for 1 min, and the supernatants were stored at −70° C until use to determine enzymatic activity. SDH activity was determined according to the method described by Fischer et al. (1985) as a decrease in the absorbance of 2,6-dichloroindophenol (DCIP) at 600 nm, with 700 nm used as the reference wavelength ($\varepsilon = 19.1$ mM^{-1} cm^{-1}), in the presence of phenazine methosulfate (PMS). For this test, the samples were thawed and refrozen three times to break the mitochondrial membranes. A reaction solution containing 40 mM potassium phosphate (pH 7.4), 16 mM succinate, and 8 μM DCIP was pre-incubated with 40–80 μg of homogenized protein at 30 °C for 20 min. Then, 4 mM sodium azide, 7 μM rotenone and 40 μM DCIP were added. The reaction was initiated by the addition of 1 mM PMS and verified after 5 min. The results were expressed as nmol/min/mg protein.

2.4.3. Complex II activity

Complex II activity was defined as a decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm, with 700 nm used as the reference wavelength ($\varepsilon = 19.1$ mM^{-1} cm^{-1}), according to Fischer et al. (1985). Immediately before this test, the samples were thawed and refrozen three times to break the mitochondrial membranes. A reaction solution containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 μM DCIP was pre-incubated with 40–80 μg of homogenized protein at 30 °C for 20 min. Then, 4 mM sodium azide and 7 μM rotenone were added. The reaction was initiated by the addition of 40 μM DCIP and verified after 5 min. The results were expressed as nmol/min/mg protein.

2.4.4. Cytochrome c oxidase activity

The activity of this enzyme was determined according to the methods described in Rustin et al. (1994). Enzymatic activity was measured at 25 °C for 10 min as a decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm, with 580 nm used as the reference wavelength ($\varepsilon = 19.1$ mM^{-1} cm^{-1}). A reaction solution was prepared containing 1 mM potassium phosphate (pH 7.0), 0.6 mM n-dodecyl-β-D-maltoside, and 2–4 μg of homogenized protein, and the reaction was initiated by adding 7.0 μg reduced cytochrome c. The results were expressed in nmol/min/mg protein.

2.5. Flow cytometry assay

2.5.1. Mitochondrial mass and membrane potential measurements

MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG) dyes were used to assess mitochondrial function. MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to create a 1 mM stock solution. The cerebral cortex samples (20 mg) were mechanically dissociated using 1 mL of phosphate-buffered saline (PBS, pH 7.4) containing 1 mg/mL of collagenase IV. These solutions were filtered through a 40-μm nylon mesh to remove large clumps of cells and debris and then stained with 100 nm MTR and 100 nm MTG for 45 min at 37 °C in a water bath in a dark room according to the method described by Keij et al. (2000) and Pendergrass et al. (2004), with some modifications. Immediately after staining, the cell suspensions were removed from the water bath and analysed using flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.5.2. Immunostaining for GFAP, NeuN and PI

Cerebral cortex samples (30 mg) were mechanically dissociated in 1 mL of phosphate-buffered saline (PBS pH 7.4), containing 1 mg/mL of collagenase IV and then filtered through a 40-μm nylon mesh to remove large clumps of cells and debris. They were then incubated in PBS/collagenase containing 10 μg/mL propidium iodide (PI). The cells were incubated at room temperature in the dark for 30 min, washed twice with PBS, and centrifuged at $1000 \times g$ for 10 min at 4 °C to remove the free PI-containing supernatant. Afterwards, the cells were permeabilized using 0.001% PBS Triton X-100 and blocked for 15 min with 1% bovine serum albumin. After blocking the cells, they were incubated for 1 h in blocking solution containing monoclonal anti-NeuN antibodies that were diluted 1:100 (Millipore Corporation, Billerica, MA, USA) or anti-GFAP antibodies that were diluted 1:100 (Dako, CA, USA). The cells were washed twice with PBS and then incubated for 1 h in blocking solution containing Alexa 488 anti-mouse IgG (diluted 1:200) or Alexa 488-anti-rabbit IgG (diluted 1:200) (Jackson Immuno Research Laboratories, Inc., PA, USA). The level of PI incorporation and the number of NeuN-positive or GFAP-positive cells were determined using flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). Alexa Fluor 488 and the PI dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples incubated with only the secondary antibody) were included in the machine voltage setup. The emission of fluorochromes was recorded using specific band-pass fluorescence filters – green (FL-1; 530 nm/30) and red (FL-3; 670 nm long pass)– and CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence emissions were collected using logarithmic amplification. Briefly, the data from 20,000 events (intact cells) were acquired, and the mean relative fluorescence intensity was determined after excluding debris events from the data set. All flow cytometric analyses were performed using Flow Jo software 7.6.3 (Treestar, Ashland, OR). Flow cytometry data were analyzed and plotted using density in dot plots, which show the relative FL1 fluorescence on the x-axis and the relative FL3 fluorescence on the y-axis. The negative and positive quadrants were determined using unstained samples. The number of cells in each quadrant was computed, and the proportion of cells stained with PI, NeuN and GFAP were expressed as percentages of the control according to the methods described in Heimfarth et al. (2012).

2.6. Oxidative stress parameters

2.6.1. Thiobarbituric acid reactive species (TBARS)

According to Draper and Hadley (1990), a test was performed to assess reactive species of thiobarbituric acid (TBARS) and lipid oxidation indices. The TBARS test consists of an acid-heating reaction containing the lipid peroxidation end product, malondialdehyde, and thiobarbituric acid (TBA). The TBARS were determined at 532 nm, and the results were expressed as nmol/mg protein.

2.6.2. Measurement of protein carbonyls

The oxidative damage to proteins was measured by quantifying the number of carbonyl groups in a reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the protocol described in Levine et al. (1994).

2.6.3. 2′,7′-Dihydrodichlorofluorescein oxidation

The production of reactive oxygen/nitrogen species was measured following the methods described in Lebel et al. (1992), which are based on analyses of DCFH oxidation and 2′,7′-dihydrodichlorofluorescein diacetate (DCFH-DA).

2.6.4. Total protein thiol content

After incubation, the swatches were analyzed for thiol content, which was used as an estimate of the number of oxidative modifications to proteins according to the methods described by Ellman (1959).

2.6.5. Antioxidant activities assays

To determine superoxide dismutase (EC 1.15.1.1) (SOD) activity, we used homogenized samples (40 μg of protein), as described by Boveris (1984). Catalase (EC 1.11.1.6) (CAT) activity was measured as previously described by Aebi (1984). Glutathione peroxidase (EC 1.11.1.9) (GPx) activity was measured according to the methods described by Wendel (1981), using tert-butyl hydroperoxide as the substrate.

2.7. Western blot analysis

Proteins (20–30 μg) isolated from cerebral cortex slices were separated using SDS-PAGE on 10% (w/v) acrylamide gels and then electrotransferred onto nitrocellulose membranes. Membranes were incubated in TBS-T (20 mmol/L Tris–HCl, pH 7.5, 137 mmol/L NaCl, 0.05% (v/v) Tween 20) containing 5% (w/v) bovine serum albumin for 1 h at room temperature. The membranes were then incubated overnight with the appropriate primary antibody, including glutathione peroxidase (1:1000; Cell Signalling®), superoxide dismutase 2 (1:5000), vitamin D receptor (1:1000; Abcam®) and β-actin (1:2000; Sigma Aldrich®). The membranes were then rinsed with TBS-T and exposed to horseradish peroxidase-linked anti-IgG antibodies for 2 h at room temperature. Chemiluminescent bands were detected using an ImageQuant LAS4000 (GE Healthcare®), and densitometry analyses were performed using Image-J® software. The results were expressed as a percentage of the control according to the technique described by de Oliveira et al. (2013).

2.8. Statistical analyses

The data are expressed as the mean ± S.E.M. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0, Chicago, IL, USA) software. Differences among groups were analyzed using two-way ANOVA followed by Tukey's post-hoc tests. $P < 0.05$ was determined to indicate significance.

3. Results

Fig. 1 shows the protective effect provided by pre-treatment with calcitriol against cell dysfunction that was induced by Hcy on brain energy metabolism and mitochondrial function. Hcy promoted a significant decrease succinate dehydrogenase-SDH activity (1A), cytochrome c oxidase (COX) activity (1B) and Na$^+$, K$^+$-ATPase activity (1C), respectively ($P < 0.05$). Fig. 1D and 1E demonstrate that Hcy caused a significant increase in mitochondrial mass (1D, $P < 0.05$) and did not affect mitochondrial membrane potentials (1E, $P < 0.05$). Pre-treatment with calcitriol (50 nM and 100 nM) blocked these effects.

In Fig. 2, we show that incubation with Hcy promoted an increase in NeuN/Pi$^+$ cells (2A, $P < 0.05$), which indicates neuronal death; however, we did not observe any difference in astrocytes (GFAP/Pi$^+$ cells) (2B). Pre-treatment with calcitriol (50 nM and 100 nM) prevented the Hcy-induced decrease in the NeuN/Pi$^+$ cells.

In Figs. 3 and 4, we sought to investigate whether pre-treatment with calcitriol would act as an antioxidant against the oxidative damage induced by Hcy. Incubation with Hcy induced a significant increase in reactive species via the oxidation of DCFH-DA (3A, $P < 0.05$), in lipid peroxidation (3B; $P < 0.05$) and in the protein carbonyl content (3C; $P < 0.05$), but did not affect sulfhydryl groups

(3D). Pre-treatment with calcitriol at 50 nM prevented these effects ($P < 0.05$). Incubation with Hcy also promoted a decrease in the activity of antioxidant enzymes (SOD activity; Fig. 4A, $P < 0.05$) and a decrease in CAT (Fig. 4B, $P < 0.05$) and GPx (Fig. 4C, $P < 0.05$). Calcitriol (50 nM and 100 nM) completely prevented these effects ($P < 0.05$).

Fig. 5 shows that Hcy did not affect SOD or calcitriol receptor-VDR immunocontent (Fig. 5A and 5B, respectively), but it did increase GPx expression (Fig. 5C, $P < 0.05$). However, pre-treatment with calcitriol at 50 nM and 100 nM prevented the Hcy-induced decrease in GPx immunocontent (Fig. 5C, $P < 0.05$) and the increase in VDR (Fig. 5B, $P < 0.05$).

4. Discussion

Elevated levels of Hcy in the circulation increase the risk of developing many neurological disorders, such as age-related diseases, Parkinson's, Alzheimer's, dementia and stroke (Manolescu et al., 2010; Obeid and Herrmann, 2006). Hcy plays a role in a shared biochemical cascade involving the overstimulation of N-methyl-D-aspartate (NMDA) receptors (Lipton et al., 1997), oxidative stress (Huang et al., 2001; Outinen et al., 1998), the activation of caspases, DNA damage and mitochondrial dysfunction (Kruman et al., 2000). However, the exact mechanisms involved in the neurotoxicity of Hcy, and the discovery of a good neuroprotective agent has not been achieved.

In the present study, we demonstrated that Hcy impaired energy metabolism, increased neuronal death and induced oxidative stress. Furthermore, pre-treatment with calcitriol (50 nM) attenuated the deleterious effects induced by Hcy, probably via the activation of vitamin D receptors, which would promote the upregulation of CBS activity (Kriebitzsch et al., 2011).

Brain energy metabolism is very important to maintain all of the physiological functions in different neural cells. Several studies have demonstrated that Hcy impairs Na+,K+-ATPase (Scherer et al., 2013), acetylcholinesterase (Scherer et al., 2014) and enzymes in the electron transport chain (Streck et al., 2003b). In agreement with previous results, we used an *ex vivo* model to show for the first time that Hcy decreased SDH, COX, Na+, K+-ATPase activity and impaired mitochondrial functionality. In addition, calcitriol prevented Hcy-induced dysfunctions in SDH, COX and Na+, K+-ATPase activity and mitochondrial swelling. Changes in enzymatic antioxidant activity and GPx levels were also prevented by calcitriol. This protective effect of calcitriol on Na+, K+-ATPase activity was previously described by Cross and Peterlik (1983) in peripheral tissues.

The toxicity of Hcy in the CNS has been extensively described to affect both neuronal survival and the ability of neurons to transmit signals and to thereby form functional neural networks. These results demonstrate that Hcy has effects that extend beyond neuronal survival (Seshadri et al., 2002). In agreement with the literature, we found in our work that Hcy promoted an increase in neuronal death but did not affect glial reactivity. In our study, we demonstrated that pre-treatment with calcitriol (50 nM) prevented neuronal death. In agreement with this result, another study showed that lower concentrations (1–100 nM) of 1,25-dihydroxyvitamin D3 provided neuroprotection against excitotoxic insults in rat hippocampal primary cultures (Brewer et al., 2001).

Several hypotheses concerning the neurotoxic effects of Hcy have been proposed, and oxidative stress is central to all of them (Petras et al., 2014). These hypotheses are in agreement with previous studies that showed that during the oxidation of Hcy, reactive species such as O_2^- and OH^-, which are harmful to most cellular components, are generated (Welch et al., 1998). In a previous pre-clinical study, our group showed that mild hyperhomocysteinemia promoted oxidative stress in the blood and the cerebral

Fig. 4. The effect of pre-treatment with calcitriol against incubation with Hcy in rat cerebral cortex slices. The effects on superoxide dismutase (A), catalase (B) and glutathione peroxidase (C) are shown. All enzymes are expressed in units/mg of protein and as the mean ± S.E.M. ($n = 6$ per group). * indicates $P < 0.05$ compared to the groups without the symbol, and # indicates $P > 0.05$ similarity to all groups, as evaluated using two-way ANOVA followed by Tukey's post-hoc test.
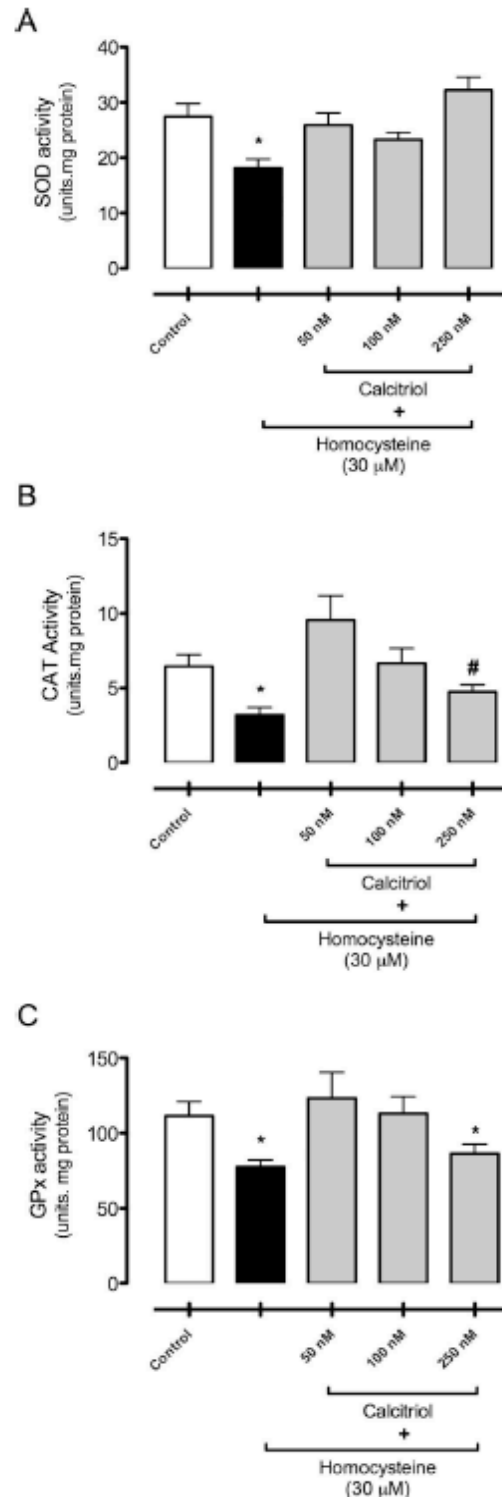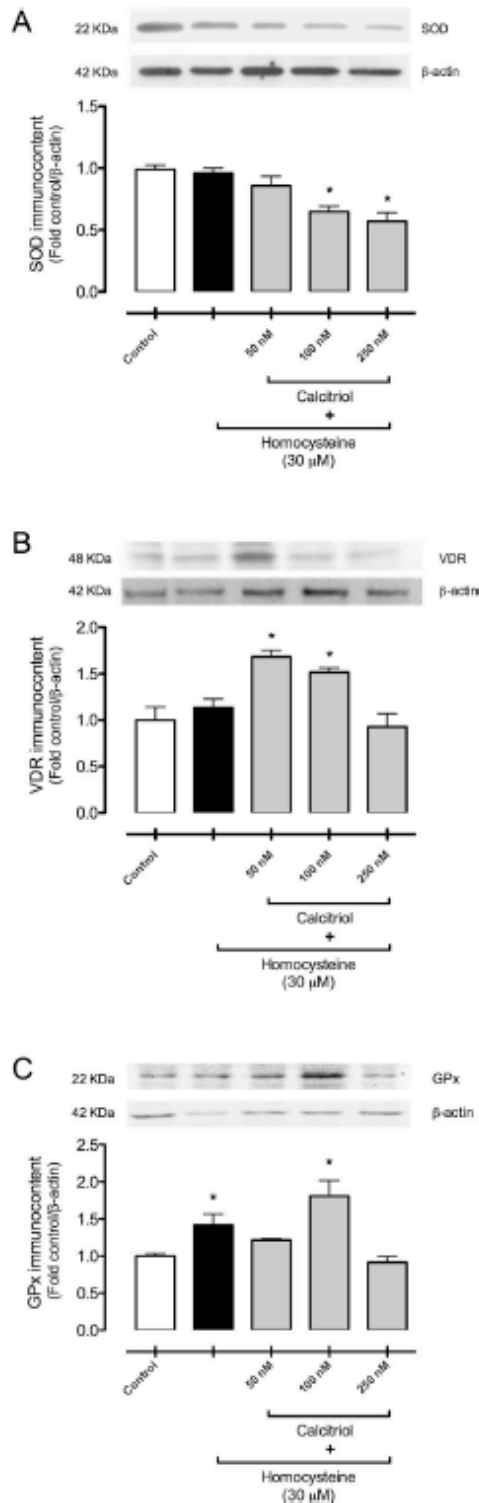
Fig. 5. The effect of pre-treatment with calcitriol against incubation with Hcy in rat cerebral cortex slices on levels of SOD (A), VDR (B) and GPx (C). The results are expressed as folds of the control/β-actin and as the mean ± S.E.M. (n = 6 per group). * indicates P< 0.05 compared to the other groups as evaluated using two-way ANOVA followed by Tukey's post-hoc test.

cortex, where it disrupted enzymatic and non-enzymatic antioxidant defences (Scherer et al., 2011b). In this study, we found that incubating cerebral cortex slices with Hcy induced an increase in the production of reactive species and in lipid peroxidation and damage to proteins, resulting in a misbalance in antioxidant enzymatic activity. We show that pre-treatment with calcitriol (50 nM) prevented these changes to the redox state. While the mechanism for the neuroprotective action of calcitriol is not clear, in the literature, recent work using the SH-SY5Y cell line showed that 1,25-dihydroxyvitamin D3 reversed the neurotoxic effects of rotenone by enhancing autophagy-related signalling pathways (Jang et al., 2014).

It is clear in the literature that calcitriol plays a physiological and very important role in the central nervous system in rodents and humans. This statement can be made based on the fact that the VDR and the enzyme (CYP27B1) responsible for the formation of calcitriol, which is the active form of vitamin D, are expressed throughout the regions of the brain (Eyles et al., 2005; Prufer et al., 1999; Veenstra et al., 1998). The receptor and the enzyme were found in both neurons and glial cells. Several clinical reports have supported an association between inadequate levels of 25-hydroxyvitamin D and increased risk of developing neurodegenerative diseases (Derex and Trouillas, 1997; Newmark and Newmark, 2007; Sato et al., 1997; Sutherland et al., 1992). Pre-treatment with calcitriol increased the level of the vitamin D receptor only when calcitriol was used at concentrations of 50 nM and 100 nM, which were the same concentrations that provided a neuroprotective effect. In agreement with our results, an extensive study by Kriebitzsch et al. (2011) that investigated the LASA cohort, a human population-based study (1264 individuals between 65 and 88 years of age), found a significant correlation between Hcy levels and 25-hydroxyvitamin D_3 levels in the plasma, with the lowest Hcy levels being observed when the 25-hydroxyvitamin D_3 level was between 50 and 60 nM (20–24 ng/mL), which is the same dose that we show promotes neuroprotective effects against Hcy toxicity. In the same study, the authors proposed that 1,25-dihydroxyvitamin D_3 promotes an increase in the mRNA levels of CBS and that functional VDR was required for this process happened. We therefore suggested that the beneficial effects of calcitriol that are demonstrated in this study are mediated through the vitamin D receptor and the up-regulation of the expression of some genes, such as CBS.

In this work, we used an in ex vivo model to show that calcitriol acts as a neuroprotective substance to prevent the alterations induced by Hcy in brain energy metabolism, the redox state and neuronal cell death. These data suggest that calcitriol may be a new target for therapies aimed at preventing the deleterious effects of mild concentrations of Hcy toxicity.

Acknowledgements

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Homocysteine induces glial reactivity in adult rat astrocyte cultures

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Abstract

Elevated concentrations of homocysteine (Hcy) are involved in neurodegenerative disorders, such as Parkinson and Alzheimer diseases. Astrocytes are a type of glial cell related to metabolic support to other neural cells, as well as detoxification of free radicals and inflammatory response. Based on this, our hypothesis was that Hcy could promote astrocytic dysfunction via NFκB signaling pathway in a model of cortical primary astrocyte culture from adult Wistar rats. Cortical astrocytes were incubated with different concentrations of Hcy (10 μM, 30 μM and 100 μM) during 24 h. After the treatment we analyzed cell viability, morphological parameters, antioxidant defenses and inflammatory response. We did not observe any alteration in cell viability; however, we demonstrated an apparent rearrangement of GFAP and actin cytoskeleton proteins. The treatment with Hcy also promoted a significant decrease in GSH content and the activities of Na^+, K^+ ATPase, SOD and GPx. Inversely the antioxidant defenses not enzymatic, the release of proinflammatory mediators were significantly increased in astrocytes treated with Hcy. In an attempt to elucidate the mechanisms involved with these findings, we measured the NFκB transcriptional activity and heme oxygenase-1 (HO-1) expression. We noted that the Hcy acted conversely in these pathways, activating NFκB and inhibiting HO-1 expression. Collectively, our results provide new evidences that Hcy activates NFκB pathway, inhibits HO-1 expression and promotes morphological, redox and inflammatory changes in adult cortical astrocyte primary cultures.

Keywords: Homocysteine, cortical adult astrocytes, oxidative stress, inflammatory response, NFκB signaling pathway.

Introduction

Homocysteine (Hcy) is an amino acid sulfur and non-proteinogenic that is formed in unequal quantities in the metabolism of essential amino acids like methionine. Hcy levels are controlled through two regulatory mechanisms: (a) remethylation, forming methionine and getting a methyl group from 5-methyltetrahydrofolate or betaine and (b) transsulfuration, when it undergoes condensation with serine, producing cystathionine, via reaction catalyzed by cystathionine β-synthase, being this product after cleaved to cysteine [1]. Hcy metabolism requires coenzymes such as vitamins B_6, B_{12} and folic acid. Deficiencies in these cofactors are associated with hyperhomocysteinemia (HHcy) that is an abnormal high level of Hcy in the blood, commonly associated to cytotoxicity. In addition, mild levels of Hcy (>30 μM) have been reported as an independent risk factor for cognitive dysfunction [2] and neurodegenerative disorders [3]. According to previous studies from our group, mild HHcy induces oxidative stress and increases neuroinflammation in the cerebral cortex of rats [4,5]. More recently we have demonstrated that Hcy (30 µM) altered mitochondrial function and induces oxidative stress and neuronal death in slices of cerebral cortex [6].

Astrocytes correspond to 50% of the total number of cells in the central nervous system (CNS), being the most versatile cells in the brain [7]. These cells have a variety of functions, including the control of neurotransmitter systems and ionic homeostasis, the regulation of metabolic functions, antioxidant defenses and inflammatory response [8]. Astrocytes have the main antioxidant enzymatic defenses, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). GPx is extremely important in the biosynthesis of glutathione (GSH), which is the major non-enzymatic antioxidant defense in the CNS [9]. Additionally, astrocytes express the enzyme Na^+, K^+ ATPase, which is crucial for maintaining the membrane potential through the active transport of Na^+ and K^+ ions in the CNS. As astrocytes are involved in the regulation of ionic homeostasis and in the glutamate transport (highly dependent of Na^+ ion), there is a close relationship between Na^+, K^+ ATPase activity and astrocyte functionality [10]. Concerning to inflammatory response, astrocytes release pro-inflammatory cytokines, such as tumor necrosis factor –

alpha (TNF-α), interleukin 1– beta (IL-1β) and interleukin – 6 (IL-6), which might be regulated by the transcription factor NFκB, the master regulator of oxidative stress and inflammation [11]. Recently, our group established a routine culture protocol of astrocyte from cortical adult Wistar rats, which presents connections more organized and well established than astrocytes derived from newborn animals, being more reliable to study the brain aging, as well as age-related neurological diseases [12]. Our studies using *in vitro* astrocytes showed classical astrocytic markers, and actively participate in antioxidant and inflammatory responses [12].

Heme oxygenase-1 (HO-1) is the inducible isoform of heme oxygenase regulated by the transcription factor nuclear factor erythroid 2 (Nrf2)-regulated gene that plays a critical role in the prevention of oxidative stress and inflammation [13]. This potential link between inflammation and HO-1 was initially demonstrated in animal models that upregulation of HO-1 attenuates the pro-inflammatory effects [14]. This effect might be attributed to ability of HO-1 inhibit the translocation of NFκB from the cytoplasm to the nucleus [13]. Additionally, HO-1 can act sequestering NO, inhibiting the synthesis of inducible oxide nitric synthase (iNOS), contributing to the control of oxidative stress [15].

The neurotoxic mechanisms of Hcy in neurodegenerative diseases have been largely studied [16-19]. However, data about the neurotoxic effect of Hcy on adult astrocytes and its mechanisms remain unclear. Furthermore, HHcy is associated to neurodegenerative disorders, thus, the adult astrocyte cultures might explain the role of HHcy in these diseases. Based on this statement, the hypothesis for this study was that Hcy could promote astrocytic dysfunction via NFκB/HO-1 signaling pathway in a model of cortical primary astrocyte cultures from adult rats. Therefore, we treated astrocyte cultures with different concentrations of Hcy and evaluated the antioxidant and inflammatory responses, as well as the putative pathways involved in these mechanisms.

Material and methods

Ethics Statement

Our work has followed the National Institute of Health Guide for the Care and Use of Laboratory Animals "Guide for the Care and Use of Laboratory

Animals" (NIH publication No. 80-23, revised 1996) and experiments were approved by the local Ethics Commission - CEUA/UFRGS), under the project number 26073.

Animal

Male Wistar rats (90 days old) were obtained from our breeding colony at the Central Animal House of the Department of Biochemistry. They were held under a normal light-dark cycle (lights on 7 h – 19 h) at room temperature (22 ± 1°C), with water and commercial food pellets available *ad libitum.* These conditions were kept constant throughout the experiments.

Cortical primary astrocyte cultures from Wistar adult rats

Cerebral cortex was dissected aseptically and the meninges removed. During the dissection, the structures were maintained in HBSS (Hank's Balanced Salt Solution) containing 0.05% trypsin and 0.003% DNAse and maintained at 37°C for 15 min. The tissues were mechanically dissociated, using a pasteur pipette and centrifuged at 400 g for 5 min. The pellets were resuspended in HBSS solution containing 40 U of papain/mL, Cysteine 0.02% and 0.003% DNAse and again gently mechanically dissociated with a Pasteur pipette. After another centrifugation step (400 g, 5 min), cells were resuspended in HBSS containing only DNAse (0.003%) and after naturally decant for 30 – 40 min. The supernatant was collected and centrifuged for 7 min (400 g). The cells from the supernatant were resuspended in DMEM/F12 10% fetal bovine serum (FBS), 15 mM HEPES, $NaHCO_3$ 14.3 mM, 1% fungizone and 0.04% gentamicin, and then plated in 6- or 24-well pre-coated with poly-L-lysine and cultured at 37°C and 5% CO_2 [20,12].

Maintenance of cell culture

The first change of medium was performed after 24 h of culture. During the first week, the medium change occurred once every two days, and from the second week, once every four days. From the third week onwards, the cells received medium supplemented with 20% FBS. Around the third to fourth week, cells reached confluence and were used for the experiments.

Treatments

After astrocytes reaching the confluence, we studied the response of these cells on Hcy. The culture medium was removed and the cells were incubated with different concentrations of Hcy (10 μM, 30 μM and 100 μM) in a DMEM/F12 with 1% FBS for 24 h at 37°C in an atmosphere with 5% CO_2.

Cellular viability – MTT reduction assay

After incubation of Hcy, MTT was added (50 ug/mL) and cells were incubated for 30 min at 37°C. Subsequently the medium was removed and the MTT crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance values were measured at 560 and 650 nm. The results are expressed as percentages of the control value.

Cellular membrane integrity – propidium iodide (PI) incorporation assay

Cells were treated simultaneously with 7.5 μM PI and incubated for up 24 h at 37°C and 5% CO_2. The optical density of fluorescent nuclei (labeled with PI), indicative of loss membrane integrity, was determined with Optiquant software (Packard Instrument Company). Density values obtained are expressed as a percentage of the control value.

Immunofluorescence

As previously described by our group [21], immunofluorescence was performed by fixing the cell cultures with 4% paraformaldehyde for 20 min and permeabilizing with 0.1% Triton X-100 in PBS for 5 min at room temperature. After a blocking overnight with 4% albumin, cells were incubated again overnight with anti glial fibrillary acidic protein – GFAP (1:400), at 4 °C, followed by washing with PBS and incubation with specific secondary antibody conjugated to Alexa 488 Fluor ® (green color) for 1 h at room temperature. For actin-labeling analyzes, the cells were incubated with 10 mg/ml rhodamine-labeled phalloidin in PBS for 45 min and two washes with PBS. Cell nuclei were stained with 0.2 mg/ml of 4',6'-diamino-2-phenylindole (DAPI). Astrocytes were analyzed and photographed with a Nikon microscope and a TE-FM Epi-Fluorescence accessory.

Determination of the activity of the Na$^+$, K$^+$- ATPase

The reaction mixture for determine the activity of Na^+, K^+ - ATPase contains 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 200 μL. After 10 min pre-incubation at 37 °C, the reaction was initiated by addition of ATP to a final concentration of 3.0 mM and incubated for 20 min. The controls were performed under the same conditions, with addition of 1.0 mM ouabain. Its activity was calculated as the difference between the two assays [22]. The free inorganic phosphate (Pi) was measured by method [23] and specific activity of the enzyme was expressed by free nmol Pi/min/mg protein.

Evaluation of antioxidant enzymes activities

The activity of SOD was determined by the method based on the ability to oxidize the pyrogallol, a process dependent of superoxide. This inhibition of auto-oxidation occurs in the presence of SOD and its activity can be analyzed by spectrophotometer at 420 nm [24]. The activity of CAT was analyzed according to the method of Aebi [25], which is based on the disappearance of H_2O_2 at 240 nm. A unit of CAT was defined as 1 μmol consumed hydrogen peroxide/minute and the CAT activity was calculated by units/mg protein. Activity of GPx was measured using tert-butyl hydroperoxide as substrate. The disappearance of NADPH was monitored at 340 nm. One unit of GPx is defined as 1 mmol of NADPH consumed/min and the activity is represented units/mg protein [26].

Glutathione content

The levels of GSH were evaluated as described by Souza et al. [12]. Homogenate astrocytes were diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was analyzed with o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using wavelengths of excitation and emission 350 nm and 420 nm. A calibration curve was performed with standard GSH solutions at concentrations ranging from 0 to 500 μM. The calculated results are expressed in nmol/mg protein.

Inflammatory response

TNF-α levels in the extracellular medium were assayed using ELISA for TNF-α from Peprotech. The levels of IL-1β and IL-6 in the extracellular medium were measured using ELISA kits from eBioscience. The results are expressed in ng/mL. The average minimum sensitivity of the ELISA kit detection is 0.4 ng/ml of cytokines [20].

NFκB levels

The levels of NFκB p65 in the nuclear fraction, which had been isolated from lysed cells by centrifugation, were measured using an ELISA commercial kit from Invitrogen (USA). The results are expressed as percentages relative to the control levels. The ELISA kit detects a minimum of 50.0 pg/mL [20].

Western blot analysis

Accordingly to the method of Souza et al. [12] with modifications, cells were homogenate using a lysis buffer solution with 4% SDS, 2 mM EDTA, and 50 mM Tris–HCl, pH 6.8. Equal amounts of proteins (45 µg) from each sample were boiled in sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% b-mercaptoethanol, 12% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and submitted to electrophoresis in 10% (w/v) SDS–polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma-Aldrich). The membranes were incubated with polyclonal anti-HO1 (1:200). GAPDH was used as a loading control. After incubating overnight with the primary antibody at 4 °C, membrane was washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:5000 for 2 h at room temperature. The chemiluminescence signal was detected using an ECL kit (Amersham), and after the films were scanned and bands were quantified using the ImageJ software (1.48v, National Institutes of Health, USA).

Statistical analyzes

Data are expressed as the mean ± S.E.M. All analyzes were performed using the Statistical Package for the Social Sciences (SPSS 16.0, Chicago, IL, USA) software. Differences among groups were analyzed using one-way ANOVA followed by Tukey's *post hoc* test, *P < 0.05.*

Results

Effects of Hcy on cell viability, GFAP and actin cytoskeleton

First, we performed the MTT assay and PI incorporation to analyze the cell viability/integrity of cortical astrocytes treated during 24 h with different concentrations of Hcy (10 µM, 30 µM and 100 µM). Figure 1 showed that Hcy did not affect the membrane integrity (1A) nor cell viability (1B). We also conduct the same analysis at different times (4 h, 8 h and 12 h) and did not observe any significant change.

Next, cells treated with Hcy for 24 h showed no significant change in nuclear morphology (Figure 2). Immunofluorescence analysis using anti-GFAP and phalloidin (for actin) was also performed to analyze the impact of Hcy exposure in the astrocyte cytoskeletal structure. At baseline, the cells presented an intense cytoplasmic immunostaining for GFAP, affecting the phenotype of astrocytes, as well as staining for actin. When cells were exposed to Hcy, we observed rearrangement of GFAP filaments and stress fiber reorganization (Figure 2).

Hcy treatment impaired Na^+, K^+- ATPase activity and antioxidant defenses in adult cortical astrocytes

Figure 3 shows that Hcy (10, 30 and 100 µM) decreased the activity of the enzyme Na^+, K^+- ATPase in adult cortical astrocytes ($P < 0.01$). Interestingly, we did not observe differences between different doses of Hcy.

Additionally, we measured the main non-enzymatic antioxidant defense, GSH, in cultured adult astrocytes and observed a significant decrease in GSH content after the exposure of 30 and 100 µM Hcy (Figure 4, $P < 0.05$).

We also measured the antioxidant enzymatic defenses in adult astrocytes and observed that 10 µM, 30 µM and 100 µM of Hcy promoted the reduction of SOD (Figure 5A, $P < 0.05$ to 10 µM, and $P < 0.001$ to 30 and 100 µM) and GPx activities (Figure 5C, $P < 0.05$ to 30 µM and $P < 0.01$ to 100 µM). Hcy did not alter CAT activity (Figure 5B).

Hcy induced inflammatory response

Changes in redox homeostasis are closely associated to the inflammatory response. To observe this relationship, the levels of classical pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, were measured (Figure 6). Hcy increased the release of these cytokines, mainly at 100 μM.

Hcy enhanced NFκB transcriptional activity and reduced HO-1 expression protein

To investigate the putative mechanisms of Hcy neurotoxicity, we measured the transcriptional activity of NFκB, the master regulator of oxidative and inflammatory responses, and the expression levels of its regulator, HO-1.

Data showed that all concentrations of Hcy increased NFκB transcriptional activity (Figure 7A, $P < 0.05$). The protein levels of HO-1, a key antioxidant/anti-inflammatory enzyme to maintain the redox homeostasis, in adult astrocyte cultures after incubation with different concentrations of Hcy were decreased in a dose-dependent manner (Figure 7B, $P < 0.05$).

Discussion

The detrimental effects of Hcy are already well documented in the literature, but the cellular mechanisms underlying Hcy-induced dysfunction needs to be better elucidated, including the effect of Hcy in glial functionality. In the present study, by the first time in our knowledge, we demonstrated that Hcy-induced changes in astrocytes morphology, as well as increased NFκB transcriptional activity and the release of pro-inflammatory cytokines, and decreased HO-1 expression levels and cellular antioxidant defenses.

Recently we demonstrated that Hcy promoted a decrease in the enzymatic antioxidant activities (SOD and GPx) and increased neuronal death in cerebral cortex slices, of rats providing evidence that this brain structure is susceptible to damage caused by Hcy [6]. There are several hypotheses relating the neurotoxic effects of Hcy to oxidative stress, due to Hcy autoxidation, which promotes an imbalance in the activity of antioxidant enzymes [27]. Our present findings corroborate with the literature, indicating that Hcy causes a significant decrease in SOD and GPx activities. SOD catalyzes the dismutation of the superoxide radical (O_2^-), forming hydrogen peroxide (H_2O_2), a reactive species less harmful that is degraded by other enzymes such GPx and CAT [28]. The decrease enzymatic activity of SOD and GPx may be caused by accumulation of reactive species, such as the O_2^- and H_2O_2, which might be toxic and provoke cellular biomolecules damage, activating signaling pathways, such as the of NFκB that may lead inflammation, energetic dysfunction/oxidative stress and, finally, cell death.

A proper operation of the enzyme Na^+, K^+-ATPase is very important for cellular physiological process and is dependent of the concentrations of Na^+ and K^+. In astroglial cells, this process is especially important, once the increases of reactive oxidative stress (ERO) production might lead to an inappropriate functioning of Na+, K+-ATPase [29,30]. In the present work, the Hcy exposure induced a decrease in the Na^+, K^+-ATPase activity, consistent with previous report [31]. We cannot exclude the idea that the Na^+, K^+-ATPase as a potent regulator of astrocytic changes, because its crucial role in brain excitability [32] and metabolic energy production [33].

GSH is an important regulator of intracellular redox state and its production might be regulated by HO-1, a signaling pathway that regulates antioxidant defenses [34,35]. In our study, Hcy decreased GSH levels and HO-1 immunocontent in the cerebral cortex, both in a dose-dependent manner, which suggest that Hcy impairs cellular defense against the ROS damage. Furthermore, the inducible HO-1 is a phase 2 enzyme upregulated in response to oxidative stress, inflammation and cellular injury [36]. Our study is the first showing the effects of Hcy on HO-1 pathway in astrocytes. Other studies in peripheral tissues demonstrated that Hcy promotes a downregulation in HO-1 expression [37,38]. Corroborating with these results, we showed that treatment with Hcy (30 µM and 100 µM) by 24 h promote a decreased in HO-1 immunocontent. This finding may indicate reduced capacity in protective mechanisms, inducing cellular damage in lipids, proteins and nucleic acids [39].

Hcy and exacerbated inflammatory response seem to be associated with the vascular dementia [40,41]. In this sense, our data showed that Hcy promoted a dose-dependent release of TNF-α, IL-1β and IL-6 from adult astrocytes. In accordance with our previous results, Scherer et al. [4] demonstrated that an *in vivo* model of mild HHcy in rats promoted an increase in TNF-α, IL-1β and IL-6 in cerebral cortex. Similarly, another study using an acute Hcy administration model in rats, demonstrated an increase in the same inflammatory markers in cerebral cortex [42]. Several studies have shown that astrocytes can respond to different stimuli, such as lipopolysaccharide, ammonia, glutamate and reactive species, releasing proinflammatory cytokines, probably by NFκB signaling pathway [43-45]. In our study, we hypothesize that the key factor for the activation of the cytokines release was the redox imbalance. In a large clinical study, Gori et al. [46] analyzed more than one thousand subjects in 2 small towns near Florence, Italy, and demonstrated that high circulating concentrations of IL-1 and IL-6 are correlate with HHcy.

NFκB is a transcription factor responsible for the activation of a number of genes and damage responses in the CNS [47]. In the cytoplasm, the NFκB p50/p65 heterodimer is inactivated through binding to IkB proteins, and the cytokines exert the opposite effect by activating the phosphorylation of inhibitory proteins by IKKs, allowing the translocation of NFκB to the nucleus to bind to

specific sequences [48]. According to our results of inflammatory markers, NFκB transcriptional activity is increased by Hcy treatment in astrocyte cultures, in a dose-dependent manner, indicating that Hcy may trigger cellular damage through NFκB pathway, which may also be associated to decrease in enzymatic and non-enzymatic antioxidant defenses as well as HO-1 expression [49-51].

Despite many advances in neuroscience, little is known about the effects of HHcy in glial functionality. Taken together, our results provide new evidences that Hcy produces deleterious effect in astrocytes leading a cellular redox imbalance, reducing antioxidant defenses, activating NFκB and promoting release of proinflammatory cytokines, which in turn, cause a energetic deficit and morphological changes (Figure 8). In summary, these results contributing to understanding the pathophysiology of Hcy, indicating possible mechanisms to be explored in the search for therapeutic agents.

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Figure Legends

Figure 1. Effects of Hcy on cell viability. Membrane integrity (a) and cell viability (b) of cortical astrocytes treated during 24 h with different concentrations of Hcy were measured as described in the Materials and Methods section. The results are expressed as % of control and represent the mean ± S.E.M. The data were analyzed statistically using a one-way ANOVA followed by a Tukey's test.

Figure 2. Cellular morphology and classical cytoskeleton markers of astrocytes. Representative cell morphology (phase contrast) and immunofluorescences of GFAP and actin with DAPI staining in cortical astrocytes after 24 h of exposure to different concentrations of Hcy. The data are three independent experiments. Scale bar 50 μm.

Figure 3. Effects of Hcy in Na^+, K^+-ATPase activity. Cells were treatment during 24 h with different concentrations of Hcy. The results are expressed as nmol Pi/min/mg protein and represent the mean ± S.E.M. The data were analyzed statistically using a one-way ANOVA followed by a Tukey's test. * indicates significant differences from basal group. **$P < 0.01$.

Figure 4. Effects of Hcy on GSH levels. Cells were treated with different concentrations of Hcy during 24 h. The results are expressed as nmol/mg protein and represent the mean ± S.E.M. The data were analyzed statistically using a one-way ANOVA followed by a Tukey's test. * indicates significant differences from basal group. **$P < 0.05$

Figure 5. Effects of Hcy on enzymatic and non-enzymatic antioxidant defenses. Cells were treated with different concentrations of Hcy during 24 h. SOD (a), CAT (b) and GPx (c) activities were measured as described in the Materials and Methods section. All results are expressed as units/mg protein and represent the mean ± S.E.M. The data were analyzed statistically using a one-way ANOVA followed by a Tukey's test. * indicates significant differences from basal group. **$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$

Figure 6. Effects of Hcy on pro-inflammatory cytokines release. Cells were treated with different concentrations of Hcy during 24 h. TNF-α (a), IL-1β (b) and IL-6 (c) levels were measured as described in the Materials and Methods section. All results are expressed as % of control and represent the mean ± S.E.M. The data were analyzed statistically using a one-way ANOVA followed by a Tukey's test. * indicates significant differences from basal group. **$P < 0.05$; **$P < 0.01$

Figure 7. Signaling pathways involved in Hcy-induced astrocyte dysfunction. NFκB p65 transcriptional activity (a) and HO-1 expression (b). All results are expressed as % of control and represent the mean ± S.E.M. of four independent experimental determinations, performed in triplicate. The data were analyzed statistically using a one-way ANOVA followed by a Tukey's test. * indicates significant differences from basal group. *$P<0.05$; **$P<0.01$

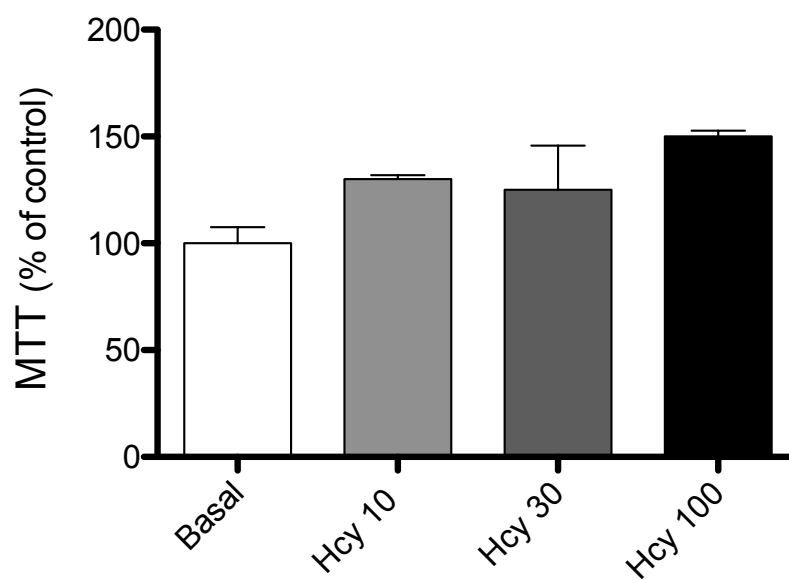**Figure 8.** Schematic illustration of the signaling mechanisms involved in Hcy-induced cytotoxicity in adult cortical astrocyte cultures.

Figure 1.

A

B

Figure 2.
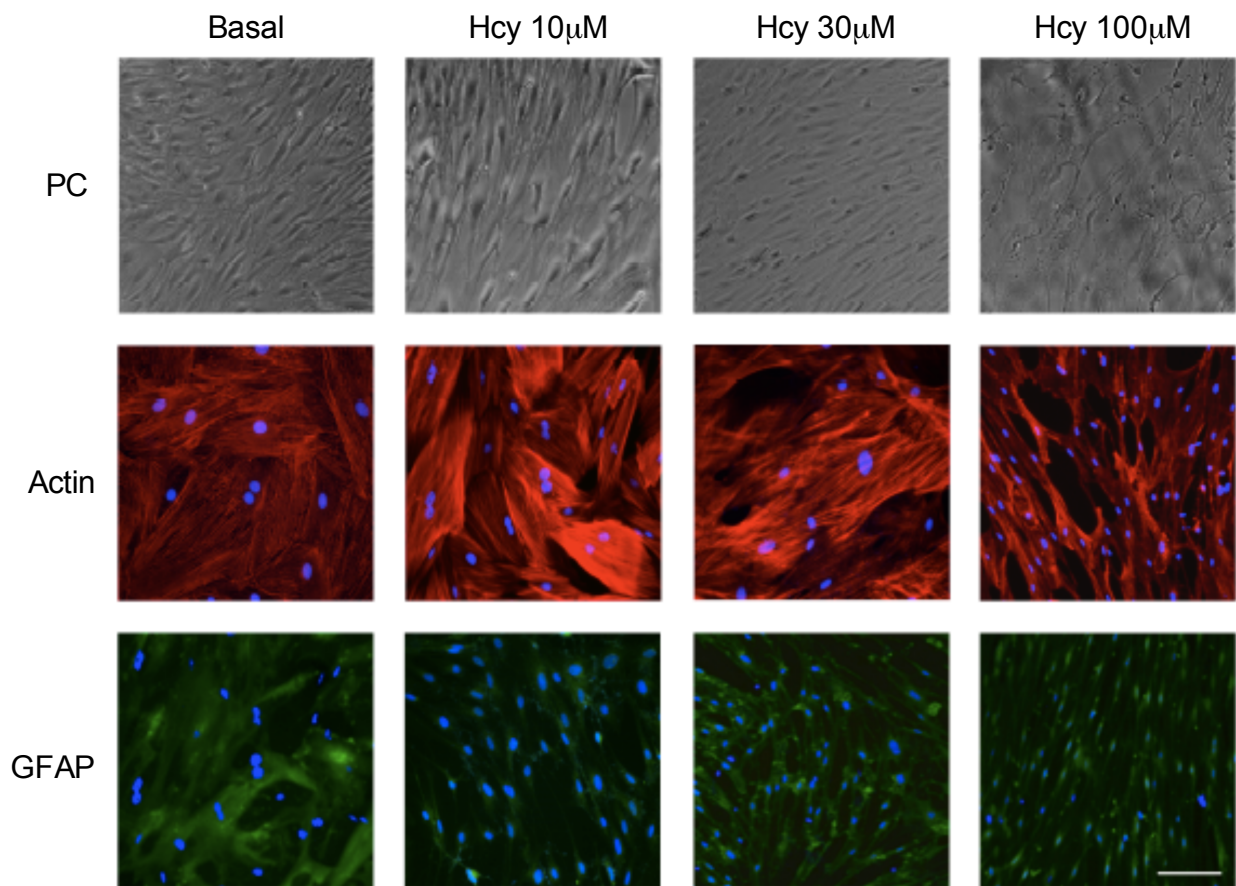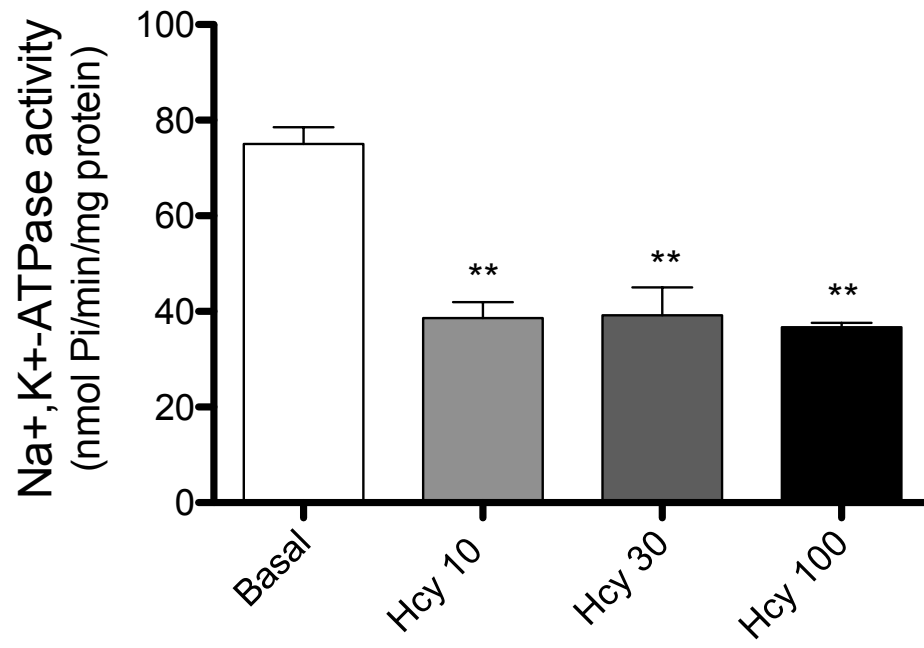

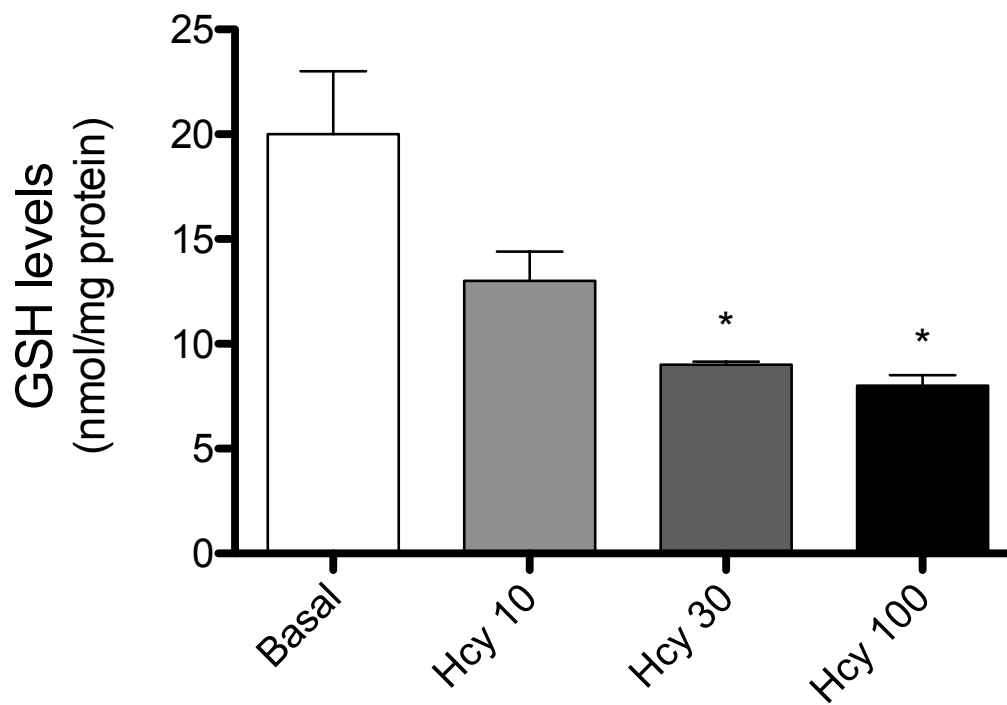|	Basal	Hcy 10μM	Hcy 30μM	Hcy 100μM
PC				
Actin				
GFAP				
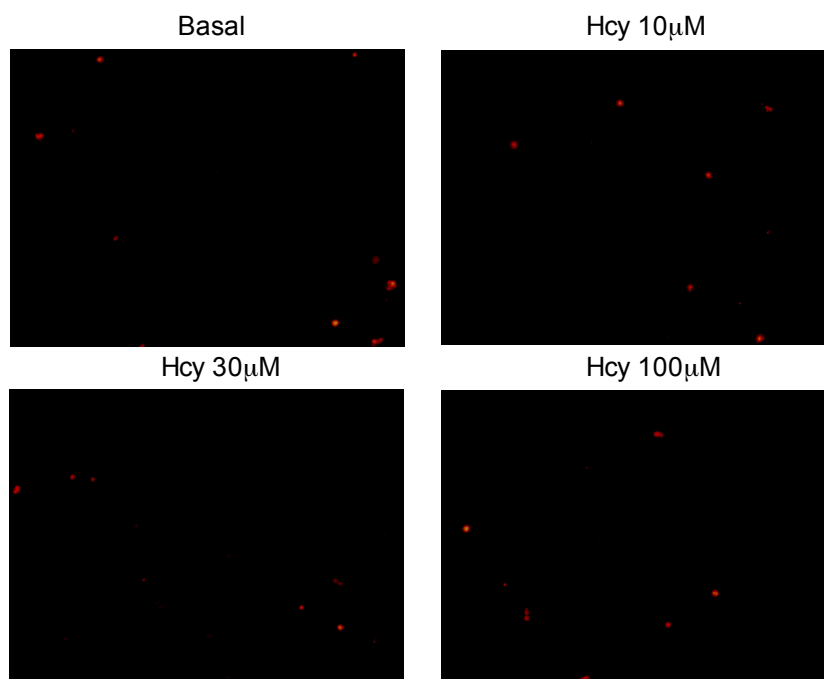
Figure 3.

Figure 4.

Figure 5.

Figure 6.

A

B

C
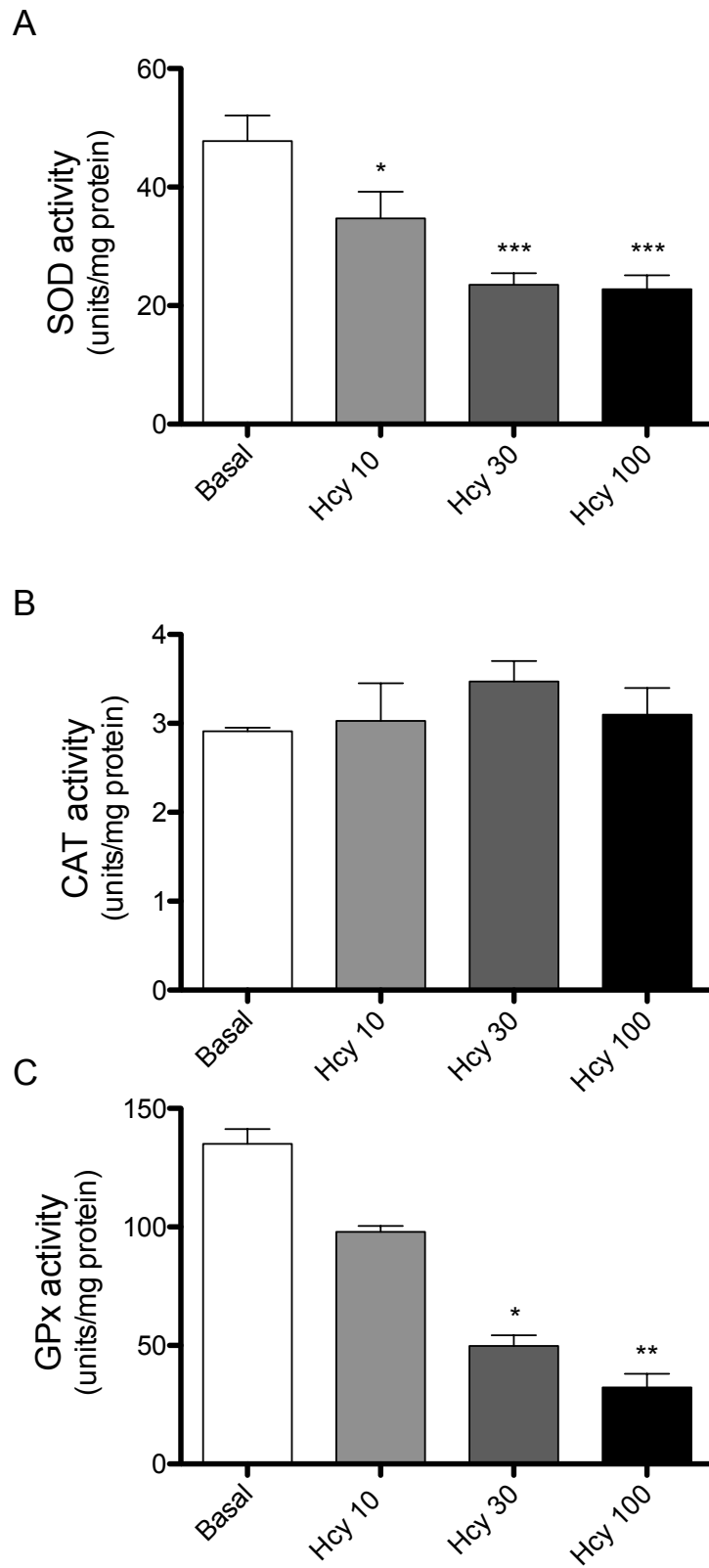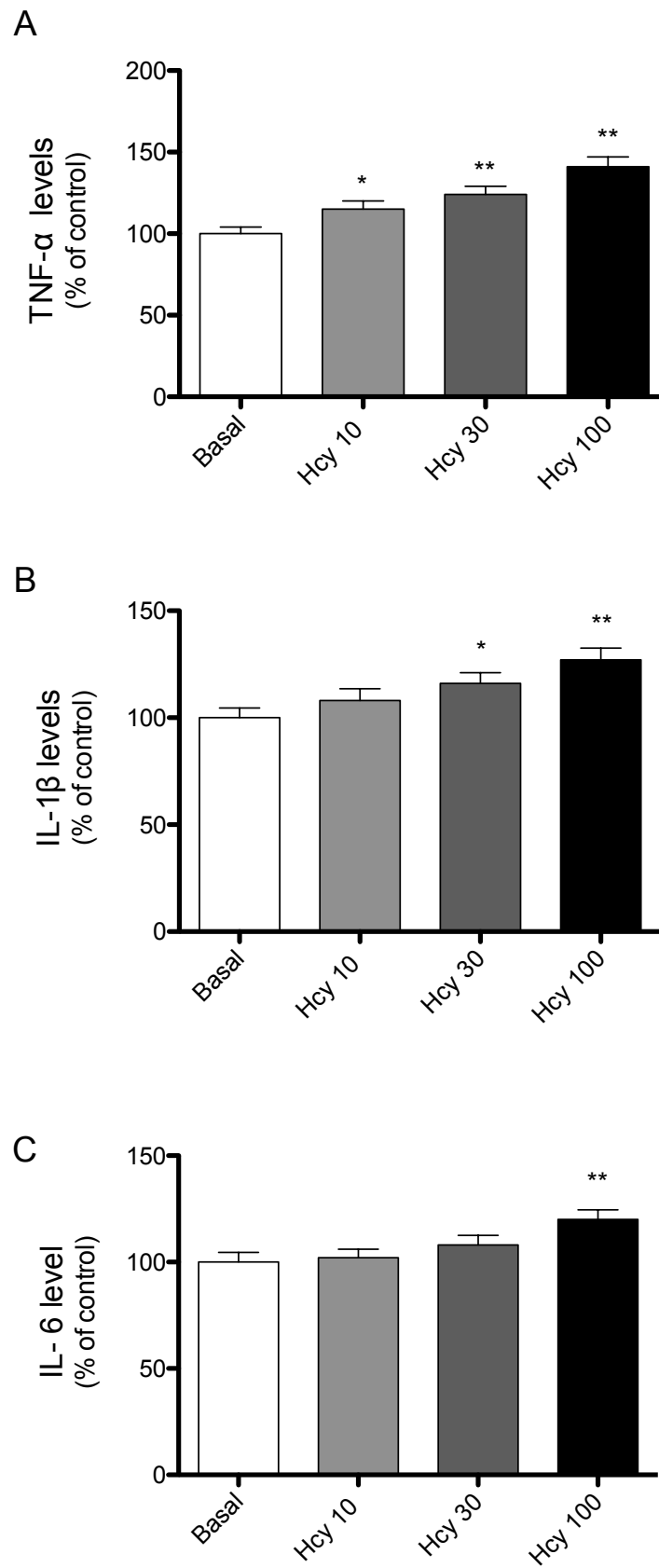
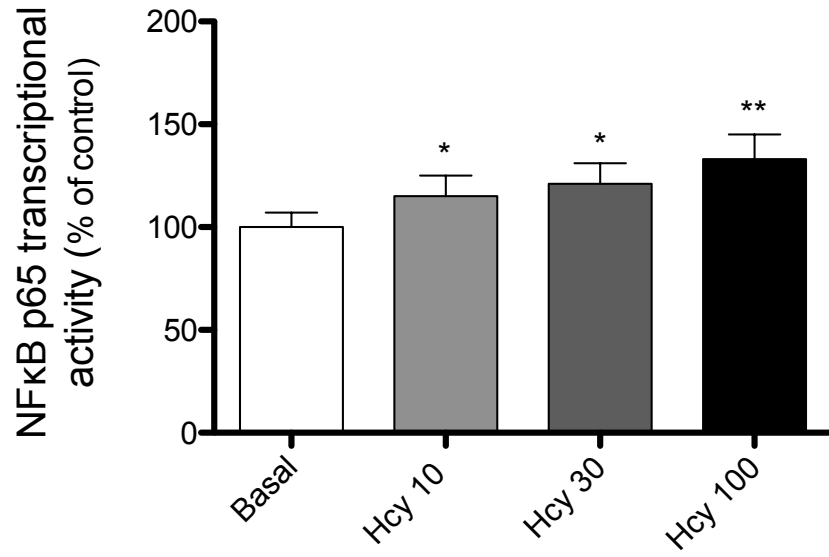**Figure 7.**

A

B
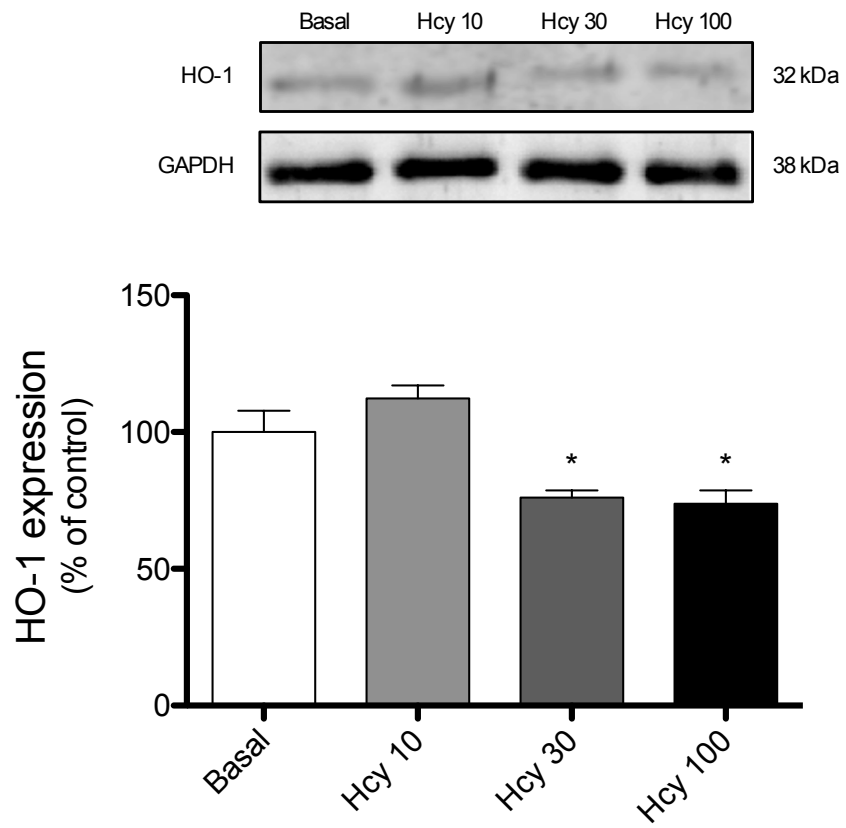
Figure 8.
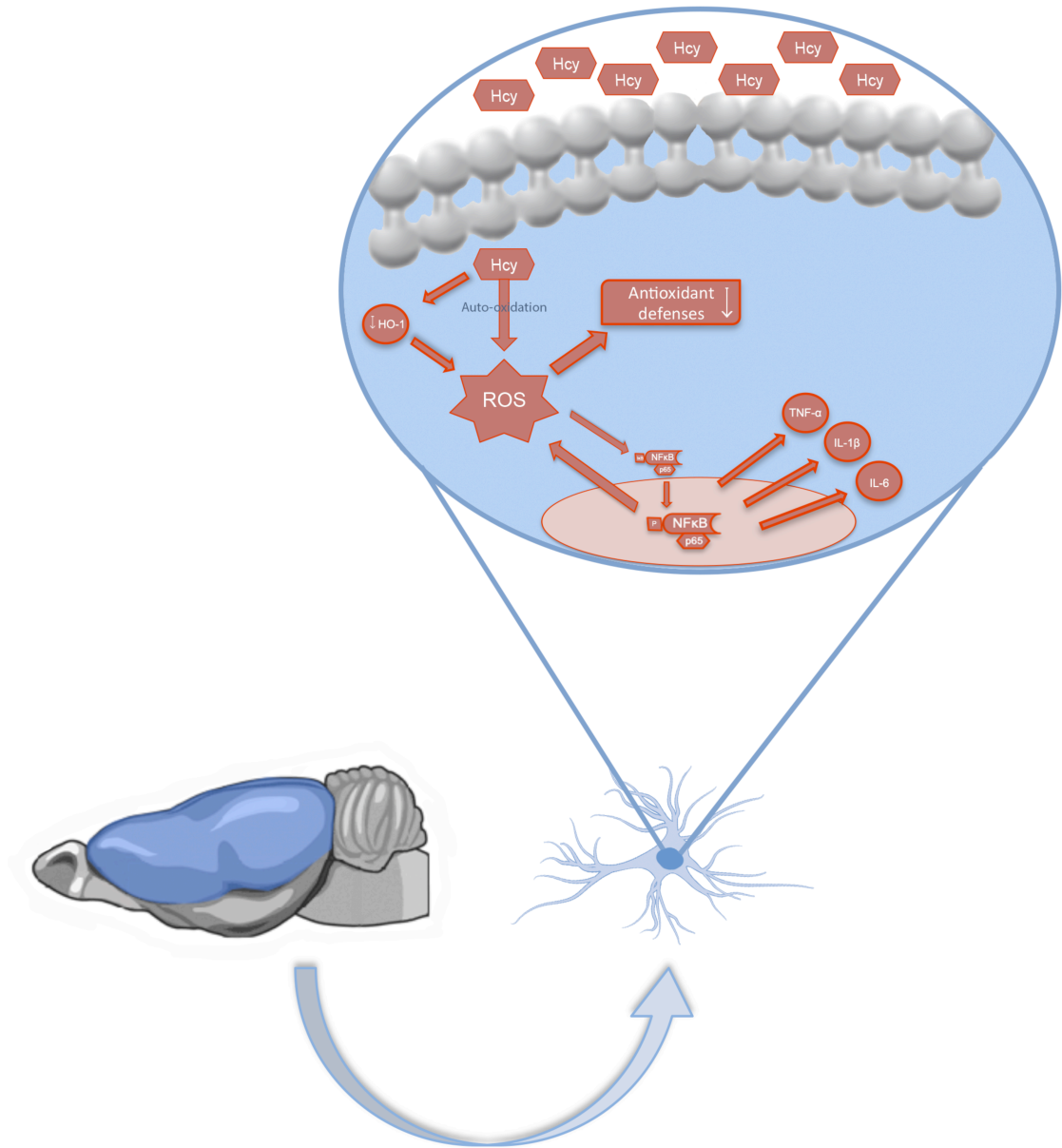
A HHcy é caracterizada pelo acúmulo de Hcy na circulação e vem sendo considerada um fator de risco para doenças cardiovasculares e cerebrais. Acredita-se que, cerca de 7% da população apresenta HHcy, uma vez que essa condição pode ser causada por diversos fatores, como, deficiências nutricionais, medicamentos, doenças renais e envelhecimento (Aksoy et al., 2006; Castro et al., 2006; Selhub, 2006; Troen et al., 2008).

Desta forma, o principal objetivo desse trabalho foi estabelecer dois modelos experimentais de HHcy leve em fatias de tecidos e em cultura primária de ratos adultos. A concentração e o tempo de incubação foram escolhidos com objetivo de induzir uma concentração de Hcy semelhante aquelas consideradas fator de risco para o desenvolvimento de doenças cerebrais e cardíacas em humanos. Analisamos também diferentes doses de vitamina D, a fim de estabelecer a melhor dose terapêutica para tentar prevenir os efeitos tóxicos da Hcy.

4.1. Experimentos em fatias com vitamina D (coração e córtex)

Níveis elevados de Hcy tem sido observados em muitas condições médicas, incluindo vários distúrbios neurológicos e cardiovasculares. A patogênese da Hcy atrai a atenção de diversos pesquisadores, simplesmente porque a intervenção precoce normalizando os níveis de Hcy pode ser benéfica para esses pacientes, prevenindo assim a toxicidade do mesmo. Entretanto, estudos clínicos demonstraram conclusões inconsistentes sobre os mecanismos de ação da Hcy (Zhu et al. 2007; Ansari et al., 2014). Utilizando um modelo experimental de incubação de HHcy leve analisamos em fatias de coração de ratos adultos parâmetros como estresse oxidativo, função

mitocondrial, parâmetros bioenergéticos, bem como o imunoconteúdo de enzimas antioxidantes e receptores de vitamina D (VDR). Embora diversos estudos demonstram a correlação entre os níveis de Hcy e os riscos de desenvolvimento de doenças cardiovasculares (Eikelboom et al., 1999; De Bree et al., 2002; Cacciapuoti, 2011) não existe uma estratégia terapêutica eficaz. Simultaneamente testamos os efeitos protetores do calcitriol, utilizando o modelo *ex vivo* envolvendo a incubação de fatias com Hcy em um pré-tratamento com o calcitriol. Porém, já está bem estabelecido que o acúmulo de Hcy no coração pode estar associado ao metabolismo mais lento, devido à deficiência na via de transulfuração, pela ausência da enzima CBS, gerando assim uma via de transulfuração incompleta (Finkelstein, 1998).

A fosforilação oxidativa mitocondrial gera radicais livres e os complexos da cadeia de transporte de elétrons são vulneráveis a esses radicais livres (Dudkina et al., 2008; da Cunha et al., 2013). Citocromo *c* oxidase é um marcador de fosforilação oxidativa, na cadeia respiratória, que catalisa um passo limitante na transferência de elétrons ao oxigênio molecular (Beal, 1992). Portanto, a inibição desta enzima pode conduzir a redução incompleta do oxigênio e, consequentemente, ao aumento da formação de radicais livres (Bose et al., 1992). A fim de elucidar como a Hcy influencia o funcionamento da cadeia respiratória, nós examinamos as atividades da SDH, complexo II e citocromo *c* oxidase. Nossos resultados mostraram que a Hcy reduz a atividade dessas enzimas, sugerindo que em baixa concentração esse aminoácido pode comprometer o metabolismo energético. O calcitriol foi capaz de reverter esses efeitos.

As disfunções mitocondriais ocorrem principalmente em função de alterações no potencial e na massa mitocondrial (Distelmaier et al., 2008). Para saber se a disfunção na atividade da cadeia respiratória foi devida a alterações mitocondriais, nós resolvemos avaliar o potencial e a massa mitocondrial por citometria de fluxo utilizando fluoróforos mitocondriais. Observamos que a Hcy induziu uma elevação na massa mitocondrial, mas este efeito não foi acompanhado por alterações no potencial mitocondrial. A transferência de elétrons através dos quatro complexos de proteína são acompanhadas por bombas de prótons da matriz para o espaço intermembrana, ocorrendo a formação de um gradiente eletroquímico, que corresponde ao potencial de membrana. Este potencial pode ser considerado um indicador importante da função mitocondrial e da atividade metabólica. Alterações nesse parâmetro pode levar a uma alteração substancial na homeostase energética celular (Solaini et al., 2007; Distelmaier et al., 2008). Além do que foi exposto, temos ainda que, o calcitriol foi capaz de prevenir estes efeitos.

Uma vez que a disfunção mitocondrial pode levar à formação de especies reativas e/ou induzir o estresse oxidativo, investigamos o efeito da Hcy sobre alguns parâmetros do estado redox em fatias de coração. Em diversos estudos já realizados, foi encontrado que este aminoácido promove um aumento na peroxidação de lipídios e na produção de ERO, bem como danos às proteínas, o qual é indicado por uma elevação no conteúdo de carbonilas e uma redução do teor de tióis (Malinow, 1990; Hogg, 1999; Cavalca et al., 2001). Os miócitos cardíacos, células endoteliais, fibroblastos e músculos vasculares são fonte celular de produção de ERO. Além disso, é bem documentado que o miocárdio tem sistemas antioxidantes enzimáticos e não-

enzimáticos endógenos que neutralizam essas ERO geradas pelo metabolismo celular.

As principais fontes de espécies reativas de oxigênio no infarto agudo do miocárdio são a cadeia de transportadora de elétrons mitocondrial, a NADPH-oxidase e/ou auto-oxidação de muitas substâncias, tais como Hcy (Misra et al., 2009). Diante disso, investigamos se as defesas antioxidantes estavam ativas no tecido cardíaco. Observamos que a Hcy causou uma diminuição nas atividades da SOD, CAT e GPX, bem como no imunoconteúdo da SOD e da CAT. Estes resultados sugerem que a Hcy promoveu um desequilíbrio oxidativo em fatias de coração de ratos, o que significa que o sistema antioxidante não foi eficaz de manter o equilíbrio oxidativo. Além disso, verificamos que o calcitriol preveniu o efeito da Hcy sobre as ERO, dano aos lipídeos e às proteínas, além de recuperar a atividades das enzimas antioxidantes e impedir a redução do imunoconteúdo dessas.

A participação do VDR mediando os efeitos cardioprotetores do calcitriol ainda é controversa na literatura (Nigwekar e Thadhani, 2013). Além disso, neste trabalho encontramos um efeito cardioprotetor do calcitriol, mas a modulação intracelular desta substância não parece ser através de VDR, porque não houve diferença no imunoconteúdo da proteína após o pré-tratamento com calcitriol. Interessantemente, o calcitriol não alterou o imunoconteúdo das enzimas antioxidantes (SOD e CAT), mas impediu a diminuição da atividade dessas enzimas. Assim, levando em consideração o modelo experimental deste estudo e os relatos encontrados na literatura (Kono et al., 2013; Wang et al., 2013), esses resultados apontam para ações benéficas do calcitriol, provavelmente promovidas por um resultado

antioxidante inibindo a sinalização pró-oxidante, sem o envolvimento da expressão do VDR.

Considerando, I) os efeitos cardioprotetores da vitamina D frente aos insultos provocados pela Hcy; II) que esses efetios não foram mediados através dos VDR, buscamos na literatura uma explicação plausível e sugerimos duas hipóteses: (1) onde esse efeito cardioprotetor foi promovido pelo coração e não pela vitamina D, insinuando que o coração foi capaz de se adaptar aos insultos promovidos pela Hcy. Concordando com essa hipótese, Nishizawa et al., 1999 demonstrou que o coração desenvolve mecanismos de adaptação em um aumento de espécies reativas; (2) que a vitamina D possa estar protegendo o coração através de um mecanismo de ação, relacionado com a inibição de γ-glutamil transpeptidase, que é a enzima chave do metabolismo da glutationa, aumentando as defesas antioxidantes, por um aumento de glutationa e assim ocorrendo uma diminuição do ERO, exercendo um efeito protetor diante os danos causados pela Hcy. Este efeito está bem estabelecido em outro órgão de acordo com o Kalueff et al., 2004.

Empregando este mesmo modelo experimental avaliamos parâmetros de metabolismo energético, funções mitocondriais e estresse oxidativo em fatias de córtex cerebral de ratos. Posteriormente, analisamos a melhor dose de vitamina D que atue prevenindo os efeitos deletérios da Hcy. Primeiramente, demonstramos que a Hcy prejudica o metabolismo energético, diminui a atividade das enzimas da cadeia respiratória, como a SDH e a COX, aumenta a morte neuronal e induz o desequilíbrio oxidativo, aumentando as ERO, a lipoperoxidação e o dano às proteínas. Esses achados mostram dados

similares a estudos anteriores onde a Hcy, sofrendo auto-oxidação, gera homocistina, dissulfetos mistos e ERO (Baydas et al., 2006). Em relação às enzimas antioxidantes, observamos uma diminuição na atividade da SOD, CAT e GPx, o que promove um desequilíbrio entre elas, uma vez que a SOD atua sobre o radical O_2^-. dismutando essa molécula em H_2O_2, que pode ser decomposta pela CAT ou GPx e assim, a ação coordenada dessas enzimas é capaz de detoxificar as espécies reativas. Além disso, o pré tratamento com calcitriol (50nM) atenuou os efeitos deletérios induzidos pela Hcy, provavelmente através da ativação de VDR, promovendo a regulação da atividade da CBS, de forma similar aos experimentos do coração.

O metabolismo energético cerebral é muito importante para todas as funções em diferentes células neuronais. Vários estudos têm demonstrado que a Hcy prejudica a atividade da Na^+, K^+-ATPase, da acetilcolinesterase e das enzimas da cadeia transportadora de elétrons. A toxicidade da Hcy no SNC tem sido extensivamente descrita, afetando tanto a sobrevivência neural, como a capacidade dos neurônios de transmitir sinais e de formar redes neurais funcionais. Nossos resultados mostram uma inibição da SDH e da COX, dessa forma, a transferência de elétrons pelos complexos da cadeia respiratória fica diminuída e com isso seguramente a transferência de energia para formar ATP é reduzida.

O cérebro necessita de fornecimento continuo de oxigênio e de glicose para manter seu funcionamento adequado, assim como várias funções celulares são dependentes de energia. Então quando ocorre uma redução a níveis críticos, uma cascata é ativada, desencadeada por uma depleção de ATP (Moro et al., 2005). Uma diminuição na atividade da Na^+, K^+-ATPase e

alterações nessa enzima podem induzir danos importantes na função cerebral, já que ela é fundamental para a atividade neural e para a captação de neurotransmissores (Blanco, 2005).

Estudos anteriores do nosso grupo de pesquisa demonstram que a Hcy inibe diretamente a atividade da Na$^+$,K$^+$-ATPase em córtex cerebral (Scherer et al., 2013). A redução na atividade da Na$^+$, K$^+$-ATPase causada pela HHcy leve foi ocasionada provavelmente por estresse oxidativo, uma vez que em outros trabalhos do nosso grupo mostram que agentes antioxidantes preveniram a diminuição de Na$^+$, K$^+$-ATPase em encéfalo de ratos submetidos à HHcy severa. Contudo, podemos sugerir que a inibição na atividade da Na$^+$, K$^+$-ATPase causada pela HHcy leve pode estar associada com a peroxidação lipídica, uma vez que essa enzima está inserida na membrana plasmática e assim qualquer alteração nesse microambiente pode prejudicar o seu funcionamento. Nossos achados demonstram que o calcitriol foi eficaz em prevenir a inibição da Na$^+$, K$^+$-ATPase causada pela Hcy. Um efeito protetor do calcitriol na atividade da Na$^+$, K$^+$-ATPase foi previamente descrito por Cross e Peterlik (1983) em tecidos periféricos, mostrou que a Hcy e a vitamina D se comportam de maneira similar, tanto no córtex como no coração de ratos.

A integridade funcional das mitocôndrias é essencial para sobrevivência celular (Snow et al., 2003). Encontramos no nosso trabalho que a Hcy promove um aumento na morte neuronal, mas não afeta a reatividade glial. Além disso, demonstramos que o pré tratamento com calcitriol (50 nM) previne a morte neuronal, do modo similar a incubação do coração. De acordo com este resultado, outro estudo (Jang et al., 2014) mostrou que em concentrações mais

baixas a 1,25 dihidroxivitamina D3 atua como neuroprotetora contra insultos exicitotóxicos em cultura primária de hipocampo de ratos.

Varias hipóteses relativas aos efeitos neurotóxicos da Hcy tem sido propostas, e o estresse oxidativo é central para todas. Estas hipóteses estão de acordo com estudos, que mostraram que durante a auto-oxidação da Hcy, espécies reativas geradas, são prejudiciais para a maioria dos componentes celulares. Verificamos que a incubação com Hcy em fatias de córtex cerebral induziu um aumento na produção de espécies reativas, peroxidação lipídica e no dano às proteínas. Corroborando, as lipoproteínas plasmáticas presentes nas membranas são essencialmente vulneráveis aos ataques de espécies reativas, que também prejudicam funções como transporte, permeabilidade, fluidez e atividade de enzimas (Gutteridge, 2001; Swapna et al., 2006). As defesas antioxidantes enzimáticas também foram investigadas. A Hcy foi capaz de diminuir a atividade das enzimas antioxidantes, como a SOD, CAT e GPx, demonstrando um desequilíbrio. O acúmulo de H_2O_2, causado por esse desequilíbrio enzimático, pode reagir com metais de transcrição pela reação de Fenton e Haber-Weiss, formando o radical $OH^{\cdot}$, que é um potente indutor de lipoperoxidação (Halliwell e Whiteman, 2004).

O pré tratamento com calcitriol (50 nM) foi capaz de evitar as alterações do estado redox. Embora o mecanismo da ação neuroprotetora do calcitriol não estar descrito na literatura, um trabalho utilizando linhagem celular, SH-SY5Y, mostra que a 1,25 dihidroxivitamina D_3 reverte os efeitos neurotóxicos da rotenona, melhorando as vias de sinalização relacionadas com a autofagia. Além disso, sabe-se que o calcitriol desempenha um importante papel fisiológico no SNC de roedores e humanos. Essa afirmação é feita com base

no fato de que o VDR e a enzima responsável pela formação do calcitriol, a forma ativa da vitamina D, está expresso em todas as regiões do cérebro. O receptor de vitamina D e a enzima 1α-hidroxilase foram encontrados tanto em neurônios como em células gliais (Veenstra et al., 1998; Prufer et al., 1999; Eyles et al., 2005).

Observamos em nosso estudo, que o pré tratamento com o calcitriol aumentou os níveis do receptor de vitamina D apenas quando o calcitriol foi usado na concentração de 50 nM e 100 nM, que são as mesmas concentrações que proporcionam um efeito neuroprotetor, e esses resultados foram contrários aos resultados encontrados neste trabalho em fatias de coração. De acordo com os nossos resultados, um estudo realizado por Kriebitzsch et al., (2011) investigou a LASA, estudo de base populacional humana com 1264 pessoas, com idade entre 65 e 88 anos, onde foi encontrado uma correlação entre os níveis de Hcy e os níveis de 25-hidroxivitamina D_3 no plasma. Foram observados níveis mais baixos de Hcy, quando os níveis de 25-dihidroxivitamina D_3 encontravam-se entre 50 e 60 nM (20-24 ng/mL), que é a mesma dose que promoveu efeito neuroprotetor contra a toxicidade da Hcy. No mesmo estudo, os autores propuseram que a 1,25-dihdroxivitamina D_3 promove um aumento nos níveis de mRNA da CBS e que o VDR funcional é necessário para este processo acontecer. Podemos ressaltar dessa forma, que os efeitos benéficos do calcitriol demonstrados neste estudo são mediados pelo VDR e pela super regulação da expressão de alguns genes.

4.2. Experimentos em cultura de astrócito

No último capítulo dessa tese, resolvemos investigar os mecanismos neurotóxicos da Hcy, pois estes efeitos sobre os astrócitos e seus mecanismos ainda permanecem obscuros e precisam ser melhor esclarecidos (Minagar et al., 2002; Szadejko et al., 2013). Atualmente, sabe-se que a Hcy está associada com doenças neurodegenerativas, desta forma a cultura de astrócitos adultos pode contribuir para a compreensão do papel da Hcy e a funcionalidade glial nestas doenças (Sudduth et al., 2013; Bonetti et al., 2016).

Com base nestas hipóteses, culturas de astrócitos foram tratadas com Hcy e foram analisadas as respostas antioxidante e inflamatória, bem como as vias envolvidas nestes mecanismos. Observamos no presente trabalho que, nos astrócitos a Hcy aumenta a atividade transcricional do NFκB promovendo uma diminuição das defesas celulares (SOD, GPx e GSH) e ativando a liberação de citocinas pró-inflamatórias; como resultado destes efeitos podemos observar alterações na reatividade glial.

Estudos anteriores demonstram que a Hcy promove uma diminuição nas atividades enzimáticas antioxidantes (SOD e GPx) e um aumento na morte neuronal em fatias de córtex cerebral, fornecendo evidências de que essa estrutura cerebral é suscetível a danos causados pela Hcy (Longoni et al., 2015). Existem várias hipóteses relativas aos efeitos neurotóxicos da Hcy relacionados ao estresse oxidativo, devido a sua auto-oxidação que promove um desequilíbrio na atividade das enzimas antioxidantes (Hogg, 1999). Nossos achados corroboram com a literatura, mostrando que o tratamento das células com Hcy causa uma diminuição significativa na atividade da SOD e da GPx. A diminuição da atividade enzimática da SOD e da GPx demonstra claramente

que as ERO podem se acumular, causando danos às macromoléculas de ativação das vias de sinalização como a do NFκB podendo conduzir a alterações morfológicas, assim como nós observamos. As células expostas à Hcy apresentaram uma desorganização das fibras de estresse indicando um rearranjo de filamentos de actina, disfunção energética e finalmente morte celular, assim como os resultados de córtex cerebral.

O funcionamento adequado da enzima Na^+,K^+-ATPase é muito importante para o processo fisiológico em que as células são dependentes de concentrações dos íons Na^+ e K^+ (Aperia et al., 2007). Em células astrogliais, essa enzima é especialmente importante e sensível aos radicais livres, uma vez que o aumento da produção de ERO levam ao seu funcionamento inadequado (Grisar et al., 1992; Chakraborty et al., 2003; Quincozes-santos et al., 2014). No presente trabalho, o tratamento com Hcy mostrou diminuir a atividade da Na^+,K^+-ATPase, corroborando com estudo realizado por (Schulpis et al, 2006). Não podemos excluir a idéia de que a Na^+,K^+-ATPase é um regulador potente de mudanças astrocíticas, em função do seu papel na excitabilidade cerebral (Sastry e Phillis, 1977) e no metabolismo energético (Mata et al., 1980). Além disso, em nosso estudo existe a possibilidade de ter ocorrido uma deficiência energética celular causada pela Hcy, pois esta enzima necessita de uma alta demanda energética para a sua atividade.

A GSH também é um regulador importante do estado redox intracelular, sendo que sua depleção esta associada com a neurotoxicidade, e a sua produção pode ser regulada por HO-1, uma via de sinalização das defesas antioxidantes (Steele et al., 2013; Brennan et al., 2015). Nosso resultados mostram que, a Hcy diminuiu os níveis de GSH e o imunoconteúdo de HO-1 no

córtex cerebral, de forma dose dependente, o que sugere que a Hcy compromete as defesas celulares contra o dano de ERO. A isoforma induzível da heme oxigenasse, HO-1, é uma enzima de fase 2, regulada em resposta ao estresse oxidativo, inflamação e lesão celular (Syapin, 2008). Esta pesquisa é a primeira que relaciona os efeitos da Hcy com HO-1 em astrócitos. Outros estudos em tecidos periféricos demonstraram que Hcy promove uma regulação negativa na expressão da HO-1 (Tan et al., 2013; Luo et al., 2014). A HO-1 é induzida como um mecanismo de proteção, como as enzimas antioxidantes, para proteger contra danos oxidativos dos lipídeos, proteínas e nucleoproteínas (Takahashi et al., 2004). Corroborando com esses resultados, mostramos que o tratamento de cultura primária de astrócitos com Hcy (30 mM e 100 mM) por 24 h promoveu uma diminuição no imunoconteúdo de HO-1.

Como descrito anteriormente, a diminuição da GSH está bem relacionada com a neurotoxicidade e a neuroinflamação. Essa diminuição do conteúdo de GSH em astrócitos favorece a resposta inflamatória, já que um estado pró oxidante ativa várias vias de sinalização que participam da síntese e liberação de citocinas pro inflamatórias (Lee et al., 2010; Currais e Maher, 2013). A Hcy e a resposta inflamatória parecem estar associados com a demência vascular (Lazzerini et al., 2007; Sudduth et al., 2013). O TNF-α executa muitas funções no SNC, além de iniciar uma cascata de ativações de citocinas como a IL-1β, como a IL-6 (Tanabe et al., 2010). Os nossos dados mostraram que o tratamento com Hcy promoveu um aumento nos níveis de TNF-α, IL-1β e IL-6 de astrócitos de córtex cerebral de adultos, mostrando ser dose dependente. De acordo com nossos resultados, Scherer et al., 2014 demonstraram que no modelo *in vivo* de HHcy leve em ratos, a Hcy promove

um aumento no TNF-α, IL-1β e IL-6 no córtex cerebral. Similarmente, uma pesquisa utilizando um modelo de administração aguda de Hcy em ratos, demonstrou um aumento nos mesmos marcadores inflamatórios no córtex cerebral (da Cunha et al., 2010). Vários estudos têm mostrado que os astrócitos podem responder aos diferentes agentes, tais como amônia, glutamato, radicais livres, ativando a liberação de citocinas pró-inflamatórias e este mecanismo e regulado por proteínas de sinalização via NFkB (Soliman et al., 2012; Santos et al., 2015).

A partir de nossos resultados, podemos supor que o fator chave para a ativação da liberação de citocinas foi o desequilíbrio redox. Em um grande estudo clínico, Gori et al., (2005) analisou mais de mil pacientes em 2 pequenas cidades perto de Florença, Itália, e demonstraram que as concentrações circulantes elevadas de IL-1 e IL-6 estão correlacionados a com a condição de HHcy moderada.

O NFκB é um fator de transcrição responsável pela ativação de inúmeros genes em respostas a danos no SNC (Jones e Thomsen, 2013). No citoplasma, o NFκB, heterodímero p50/p65 são inativados através da ligação de proteínas para IkB, e as citocinas exercem um efeito oposto através da ativação da fosforilação de proteínas inibidoras IKKS e sua degradação, permitindo a translocação do NFκB para o núcleo e assim se ligando a sequências específicas (Abraham, 2000). De acordo com os nossos resultados, a atividade de NFκB é aumentada pelo tratamento com Hcy em cultura primária de astrócitos, de forma dose dependente, indicando que uma resposta aos danos por via de NFκB foi desencadeada. Neste contexto, nós observamos que Hcy induz uma depleção de todos os agentes de proteção, como as

defesas antioxidantes enzimáticas e não enzimáticas e HO-1, esta redução pode estar correlacionada com o aumento do NFκB e citocinas pró-inflamatórias.

Sabendo da importância do uso de modelos experimentais para investigar mecanismos envolvidos em doenças humanas e novos alvos terapêuticos preventivos, destacamos a relevância do estabelecimento dos modelos utilizados na presente tese para estudar alterações teciduais causadas pela Hcy.

Em conjunto, os resultados desta tese demonstraram que a Hcy induz alterações no metabolismo energético, como disfunção mitocondrial, desequilíbrio no estado redox e inflamatório central e periférico, prejudicando a funcionalidade de importantes enzimas, e morte celular. Observamos que o calcitriol consegue prevenir muitos desses efeitos nocivos da Hcy via VDR e por ações antioxidantes. Desta forma, considerando que a HHcy leve é tipo mais prevalente na população e é considerada um fator de risco para doenças cardiovasculares e cerebrais, os presentes modelos experimentais podem ser úteis para maiores investigações dessas doenças e seus mecanismos, além de elucidar estratégias protetoras da vitamina D, prevenindo os efeitos deletérios da Hcy, podendo ser considerada como um possível adjuvante terapêutico.

IV.CONCLUSÕES

Nossos estudos, usando um modelo com concentrações de Hcy mostraram que à Hcy causou os seguintes efeitos:

a) alterações na função mitocondrial e no estado redox em fatias de coração, o que pode estar relacionado com complicações cardiovasculares. O calcitriol foi capaz de reverter essas alterações, protegendo contra a maioria dos efeitos da Hcy. Ressaltando que nosso estudo é o primeiro a analisar o efeito de Hcy em combinação com pré-tratamento de calcitriol no modelo agudo de fatias coração. Certamente, mais estudos ainda são necessários para elucidar os mecanismos pelos quais o calcitriol age.

b) alterações no metabolismo energético, a morte celular e o estado redox em cortex cerebral. Neste modelo *ex vivo* mostramos que o calcitriol atua como uma substância protetora, evitando essas alterações, sugerindo que o calcitriol pode ser um novo alvo para terapias destinadas a prevenir os efeitos deletérios de toxicidade da Hcy em concentrações leves.

c) alterações em astrócitos levando a um desequilíbrio no estado redox, diminuição nas defesas celulares (antioxidantes enzimáticos e não enzimáticos), ativando NFkB, promovendo a liberação de citocinas pró-inflamatórias, causando um déficit energético e alterações morfológicas via HO-1. Estes resultados contribuem para a compreensão da fisiopatologia da HHcy leve e moderada indicando um possível mecanismo para ser explorado na busca de um alvo terapêutico.

V. PERSPECTIVAS

1. Investigar por qual mecanismo a vitamina D exerce efeito neuro e cardio protetor;

2. Compreender o efeito da vitamina D em cultura de astrócitos expostos a Hcy;

3. Avaliar o efeito da Hcy em outros tecido cerebrais, hipocampo, bem como o efeito neuroprotetor da vitamina D;

4. Realizar um tratamento *in vivo* em modelo experimental de Hcy, testando o possível efeito protetor da vitamina D, bem como realizar cultura de astrócitos adultos destes mesmod animais.

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