UNIVERSIDADE FEDERAL DE MINAS GERAIS
Faculdade de Medicina

"Bloqueio do Receptor AT1 da Angiotensina II na Atividade Basal da AMP- proteína Quinase Hipotalâmica e Após Estresse de Contenção ou Cirúrgico".

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“Bloqueio do Receptor AT1 da Angiotensina II na Atividade Basal da AMP- proteína Quinase Hipotalâmica e Após Estresse de Contenção ou Cirúrgico”.

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Aos meus pais, Dorcimar e Lícia,
com todo o amor desta vida.

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CONSIDERAÇÕES INICIAIS

AMPQ - História e Relevância

A 5'-monofosfato-adenosina proteína quinase ativada (AMP-Q) foi descoberta há mais de 3 décadas como uma proteína quinase de atividade associada a acetil-CoA carboxilase[1] e HMG CoA redutase[2], enzimas de papel essencial na síntese de ácidos graxos livres e colesterol. Posteriormente, descobriu-se que AMP-Q é ativada por AMP[3] e foi reconhecido que a AMP-Q era, por sua vez, regulada pelo nível de energia celular[4] e por fosforilação.[5,6]

Após estas descobertas, seguiu-se um intervalo de tempo durante o qual a AMP-quinase foi vista apenas como uma curiosidade, sendo reconhecida a sua ativação por condições extremas de estresse metabólico, como aquelas que "desligam" as vias anabólicas para conservar energia[7].

A AMP-Q tornou-se proeminente pela descoberta de que é a responsável pela fosforilação de numerosas proteínas envolvendo funções celulares em resposta ao exercício físico e ao uso de drogas antidiabéticas como a metiformina. Além disso, a AMP-Q é a possível mediadora de muitos dos benefícios para a saúde promovidos pelo exercício físico, tais como mitigar a obesidade, a resistência à insulina e o diabetes do tipo II[8,9].

Sendo assim, a AMP-Q é reconhecida atualmente como sensor metabólico celular multifuncional, assim como um sensor de estresse funcionalmente conservado durante toda a evolução eucariótica[10,11].

Ativação do Sistema AMPQ

O sistema AMP-quinase pode ser ativado por estímulos fisiológicos, como o jejum e o exercício físico, e patológicos como a isquemia, a hipoxemia e a hipoglicemia[12,13]. A AMPQ é sensível a reduções de adenosina trifosfato (ATP) e ultra sensível a aumentos de adenosina monofosfato (AMP)[14], mas também pode ser ativada por glicogênio, e por drogas, hormônios e estresses não relacionados à redução de energia[15]. Quando ativada (fosforilada), a AMPQ deflagra os processos de restauração de energia (anabolismo), enquanto inibe os processos consumptivos (catabolismo)[15]. Isquemia, hipóxia, exercício, jejum, envenenamento metabólico (arsênico, oligomicina), estresse oxidativo, hipoglicemia e estresse osmótico são reconhecidamente fatores que ativam/fosforilam a AMP-quinase[12,13;16-20].

A ativação da AMP-Q *in vivo* é complexa, e pode variar de acordo com o estressor, sua intensidade e o órgão ativado. Por exemplo, sabe-se que a fosforilação da AMPQ cerebral ativa os receptores $GABA_B$ centrais, promovendo redução da atividade sináptica neuronal e redução do consumo de oxigênio cerebral[21]. Entretanto, a ativação excessiva da AMPQ cerebral pode ser mais deletéria do que neuroprotetora[22]. Isto se deve provavelmente ao fato de que astrócitos e neurônios utilizam glicose de forma diferente. Após isquemia cerebral, ou seja, em condições hipóxicas ou anóxicas, a AMPQ ativada aumenta a atividade da enzima 6-fosfofruto-2-quinase/frutose 2,6-bifosfatase, isoforma 3 ($PFKPB_3$) dos astrócitos, que produzem lactato a partir de piruvato, obtendo ATP suficiente para a manutenção do potencial de membrana mitocondrial ($\Delta\Psi m$) e evitando apoptose espontânea. Em contrapartida, nos neurônios, a enzima PFKFB é continuamente degradada e quase inexistente, inviabilizando a via glicose-piruvato-lactato e tornando os neurônios sujeitos a apoptose imediatamente após o início de isquemia [23,24]. Portanto, definir a intensidade de ativação de AMPQ capaz de promover proteção cerebral durante isquemia é tarefa a ser conduzida em um modelo *in vivo* com relevância clínica.

O Sistema Renina-Angiotensina

O sistema renina-angiotensina atual é descrito como um modelo duplo, no qual importa o equilíbrio entre a ativação da ECA-angiotensina II- receptor AT1 e a ECA 2-angiotensina 1 a7-receptor Mas (Fig.1)[25].

Recordando o SRA, temos o angiotensinogênio como componente comum inicial, que sofre ação da renina, sendo transformado em angiotensina I. A partir daí, a ECA transforma a angiotensina I em angiotensina II que, agindo no receptor AT1 produz vasoconstrição, fibrose e estenose vascular, aterosclerose e, por aumento de aldosterona, retenção de sódio e água. Além disto, ainda no SRA, a ECA também converte a angiotensina 1-9 em angiotensina 1-7, e a angiotensina 1-7 em angioensina 1-5. O receptor AT2 da angiotensina II interage com o receptor AT1, mas muitas de suas ações ainda são desconhecidas. Fora do SRA, a ECA degrada a bradicinina, um potente vasodilatador pulmonar.

De forma diversa, a ECA 2 catalisa a conversão de angiotensina II em angiotensina 1-7 e a angiotensina I em angioensina 1-9. Então, o heptapeptídeo angiotensina 1-7 liga-se aos receptores Mas, produzindo ações antagônicas à angiotensina II, tais como vasodilatação, redução de proliferação, hipertrofia e fibrose vascular[25].

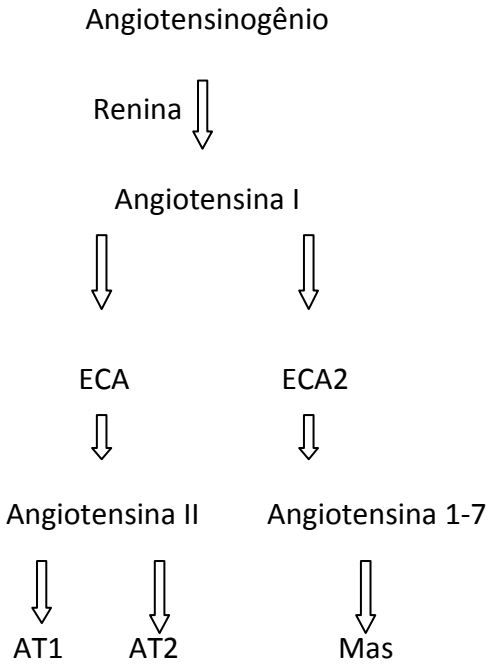

Fig. 1 O sistema Renina-Angiotensina

A População Cirúrgica Atual

Em anos recentes, a comunidade anestesiológica tem-se preocupado com o crescente aumento do número de pacientes submetidos a cirurgia geral eletiva em uso de bloqueadores dos receptores AT1 da angiotensina II[26].

Os bloqueadores AT1 da angiotensina II podem reduzir a pressão arterial por suas ações vasodilatadoras, evitar novos diagnósticos de diabetes por redução da inflamação de ilhotas pancreáticas, e evitar a progressão de nefropatia e retinopatia em diabéticos por suas propriedades antiinflamatórias e antiproliferativas[27-30].

O aumento do número de pacientes em uso de bloqueadores da angiotensina II se justifica pela elevada prevalência global de hipertensão arterial, pela epidemia de diabetes de 2000 a 2030, e também pelo fato de pacientes cada vez mais idosos serem submetidos a cirurgias eletivas de grande porte [31,32].

Além disto, houve um aumento das indicações de uso dos bloqueadores AT1 da angiotensina II, que são usados para tratamento de hipertensão arterial sistêmica, hipertensão associada à diabetes e diabetes em pacientes normotensos.

Até o presente, conhecem-se bem os efeitos hemodinâmicos dos bloqueadores de receptores AT1 da angiotensina II, mas nada se sabe sobre os seus efeitos metabólicos no período perioperatório ou seu impacto sobre a recuperação dos pacientes.

As Teorias do Estresse e o Estresse Perioperatório

Quando, em 1936, Hans Selye escreveu para a Nature descrevendo ulceração gastrointestinal, involução timicolinfática e alargamento adrenal como uma tríade patológica associada ao estresse desencadeou inúmeras pesquisas, debate e controvérsia que persistem até hoje[33].

De acordo com sua teoria, a resposta ao estresse é generalizada, não-específica e estereotipada, consistindo na ativação do eixo hipotálamo-hipófise-adrenal (HHA) e produzindo a "Síndrome Geral da Adaptação" com suas fases de alarme, resistência e exaustão[34].

A seguir, foram realizados diversos experimentos que associaram exposição ao frio, hipertermia e ortostatismo ao aumento de norepinefrina, hemorragia ao aumento de epinefrina, modificações do sódio da dieta ao aumento de angiotensina II e aldosterona, contenção ao aumento de ACTH, epinefrina e norepinefrina, entre outros[35]. Estes experimentos sugeriam respostas individuais, específicas aos diversos tipos de estresse.

Estas respostas específicas não foram ignoradas por Selye, que propôs que após subtração das respostas específicas aos diferentes tipos de estresse resta sempre o cerne, a resposta inespecífica compartilhada que caracteriza a "Síndrome Geral de Adaptação".

Como teoria alternativa, que não comprova e nem refuta a Teoria da Não-especificidade de Selye, surgiu então a Teoria da Especificidade Primitiva que advoga que a cada tipo de estresse compete uma resposta específica, com ativação de seu circuito cerebral característico e assinatura neuroquímica própria.

No caso do nosso experimento, decidiu-se estudar a cirurgia geral de grande porte, por se tratar de estresse comum na prática clínica, mas contemplando as suas fases de ansiedade pré-operatória e cirurgia sob anestesia geral *per se*. Como mimetizador da ansiedade pré-operatória elegeu-se o estresse de contenção ou imobilização e, como agravo cirúrgico de grande porte, cirurgia com abertura de grande cavidade – laparotomia com exposição de vísceras abdominais.

Objetivo do Projeto "Bloqueio do Receptor AT1 da Angiotensina II na Atividade Basal da AMP- proteína Quinase Hipotalâmica e Após Estresse de Contenção ou Cirúrgico"

A literatura científica de 2006 a 2010 vem clamando que há uma escassez de estudos *in vivo* da AMPQ ativada em cenários clínicos comuns que representem estresse[36]. Como anestesiologista, me chama atenção o fato de que a modulação (aumento, redução, ausência de alteração) da AMP-Q durante o estresse cirúrgico sob anestesia geral não é conhecida, tampouco o impacto deste estresse comum na prática clínica sobre o sistema AMP-Q em vigência do uso de bloqueador de receptor AT1 da angiotensina II.

Portanto, o principal objetivo deste trabalho é descrever, pela primeira vez, o impacto do bloqueio do receptor AT1 da angiotensina II sobre a AMPQ ativada na ausência de estresse, e também durante o estresse cirúrgico decomposto em ansiedade pré-operatória (contenção) e laparotomia sob anestesia geral.

“Bloqueio do Receptor AT1 da Angiotensina II na Atividade Basal da AMP- proteína Quinase Hipotalâmica e Após Estresse de Contenção ou Cirúrgico”.

Resumo

A AMP-proteína quinase ativada (AMPQ) tem um papel proeminente como sensor metabólico e de estresse, assumindo papéis peculiares de acordo com a magnitude do agravo, a especificidade do estressor e o órgão estudado. A AMPQ se expressa em várias áreas do cérebro, incluindo o hipotálamo, e foi demonstrado que a AMPQ hipotalâmica desempenha um papel importante em estresse e metabolismo. Os bloqueadores do receptor AT1 da angiotensina II (ARA) têm sido largamente empregados em diabetes, uma condição conhecida por suas modificações no sistema AMPQ, mas pouco é sabido sobre suas relações com a AMPQ tanto em condições basais quanto em eventos estressantes. Este estudo tem como objetivo avaliar a AMPQ hipotalâmica basal e após contenção ou estresse cirúrgico sob anestesia geral (quetamina associada com xilazina). Ratos Wistar machos foram cronicamente tratados com candesartan 5mg/Kg/dia na água de beber, ou água sem adições, por 2 semanas. AMPQ hipotalâmica basal e após contenção ou estresse cirúrgico foram determinados por imunoprecipitação. Observamos que a atividade da AMPQ hipotalâmica é aumentada pela administração crônica de candesartan em animais não estressados ($p<0.05$); e aumentada durante contenção ($p<0.01$), mas não é influenciada pelo estresse cirúrgico sob anestesia; e tratamento crônico com candesartan previne aumento subsequente na atividade da AMPQ na contenção e reduz a atividade da AMPQ no estresse cirúrgico sob anestesia aos valores observados em controles não estressados ($p<0.01$). Estas modificações mostram que ARA pode alterar os valores de AMPQ *per se* e durante ansiedade da contenção e estresse cirúrgico sob anestesia. Portanto, ARA parece modular a AMPQ hipotalâmica, e isto deve ser mais estudado para possivelmente melhorar o êxito cirúrgico.

Palavras-chave: AMPQ, bloqueador do receptor de angiotensina II, estresse cirúrgico, contenção, hipotálamo.

Implicação

O tratamento crônico com o bloqueador do receptor AT1 da angiotensina II (ARA) aumenta os valores basais da atividade hipotalâmica da AMPQ, previne o aumento de AMPQ na contenção e reduz a AMPQ no estresse cirúrgico sob anestesia geral. Estas modificações mostram que ARA pode alterar a AMPQ hipotalâmica ambos durante ansiedade e cirurgia sob anestesia. Portanto, a AMPQ pode ser um futuro alvo para possivelmente influenciar o resultado cirúrgico.

Introdução

A AMP-proteína quinase ativada é uma quinase heterotrimétrica da família serina-treonina encontrada em todas as células eucariotas[10]. Tem um papel proeminente como sensor metabólico e de estresse, assegura sobrevivência sob estresse, e foi conservada durante toda a evolução[11]. A ativação da AMPQ *in vivo* é complexa e pode assumir papéis peculiares de acordo com a magnitude do agravo, a especificidade do estressor e o órgão estudado[15]. O cérebro é considerado o órgão-chave do estresse, alvo e efetor, e a AMPQ se expressa significativamente em várias áreas cerebrais, especialmente no hipotálamo [37,38].

O sistema AMPQ tem sido relacionado a síndrome metabólica e diabetes, e os bloqueadores de receptor AT1 da angiotensina II têm um papel protetor nesta população, mesmo quando não há hipertensão[39,40]. Na prática anestésica, o número de pacientes cronicamente tratados com bloqueadores AT1 candidatos eletivos a cirurgia geral vem aumentando[26] e o papel destes agentes na medicina perioperatória não foi completamente determinado. Além disso, sabe-se que a AMPQ também pode ser ativada por certas drogas, hormônios, e estressores celulares que não alteram a relação AMP/ATP.

A AMPQ é ativada em circunstâncias de depleção de ATP e aumento de AMP, sendo considerada um ultra sensor de AMP, de tal forma que aumentos de AMP de até seis vezes são suficientes para que a atividade da AMPQ aumente de 10% para 90%[14]. Entretanto, a ativação da AMPQ *in vivo* foi pouco estudada em cenários clínicos comuns, como a ansiedade pré-operatória e a cirurgia sob anestesia geral.

Decidiu-se, portanto, avaliar a atividade da AMPQ hipotalâmica em condições basais, e após ansiedade (contenção) ou cirurgia de grande porte em ratos cronicamente tratados com candesartan, a fim de obter dados sobre o papel do sistema AMPQ nestes dois procedimentos de estresse *in vivo*, assim como a influência do bloqueador AT1 de angiotensina II na modulação da AMPQ hipotalâmica.

Materiais e Métodos

Ratos Wistar machos de 7 a 9 semanas providos pela Universidade Federal de Minas Gerais, Centro de Facilidade Animal da Faculdade de Medicina, foram mantidos sob condições controladas de temperatura e ruído, com um ciclo de luz-escuridão artificial de 12 horas, ingerindo ração standard e água *ad libitum*. Os ratos foram alojados em gaiolas individuais para permitir o controle da ingestão de líquidos, e manuseados diariamente.

Tratamento- Os ratos foram tratados com candesartan cilexetil (AstraZeneca, Cotia, São Paulo) 5mg/Kg/dia na água de beber por 2 semanas, ou com água sem adição pelo mesmo período, de acordo com protocolo previamente descrito[41,42].

O Comitê de Ética para Experimentação Animal da Universidade Federal de Minas Gerais aprovou todos os procedimentos (protocolo 110/2007).

Desenho Experimental:

Protocolo 1: Efeito da administração prolongada de candesartan sobre a ingestão de água e o peso corporal.

A fim de averiguar a aderência à ingestão da solução de candesartan, a ingestão diária de líquidos e o peso corporal de 20 ratos tratados com candesartan e 20 controles foram registrados durante duas semanas. O ganho de peso foi, então, calculado para cada grupo de animais.

Para os experimentos dos protocolos 2 e 3, os ratos foram transferidos para sala de experimentos equipada cedo na manhã do dia do experimento onde ficaram em repouso por 60 minutos em suas gaiolas. Ruído mínimo foi tolerado nesta sala de temperatura controlada e os experimentos foram realizados de 8 às 12H.

Protocolo 2: Efeitos da administração de candesartan a longo prazo sobre a atividade basal de AMPQ e após estresse de contenção.

Após 60 min de repouso em suas gaiolas, os animais foram colocados dentro de um tubo de plástico (21 cm comprimento, 4,5 cm diâmetro) onde eles não podiam se mover, causando intenso desconforto sem dor aparente, por 20minutos. Este experimento foi usado para mimetizar a ansiedade pré-operatória.

Protocolo 3: Efeitos da administração a longo prazo de candesartan sobre a atividade basal da AMPQ e após estresse cirúrgico.

Após 60 minutos de repouso nas suas gaiolas, os animais foram anestesiados através da administração intraperitoneal de quetamina (Ketamin S, Cristália, Itapira, São Paulo, Brazil) 0,075mg/g associada a xilazina (Coopazine, Schering-Plough Coopers, Cotia, São Paulo, Brazil) 0,01 mg/g. Este protocolo de anestesia foi aprovado pelo

Comitê de Ética para Experimentação Animal da Universidade Federal de Minas Gerais. Após 5 minutos, quando os ratos estavam profundamente anestesiados, foi realizada laparotomia mediana extensa, seguida por exposição dos intestinos durante 20 minutos.

Decapitação e remoção do hipotálamo

Eutanásia foi realizada através de decapitação no estado basal ou após 20 minutos de estresse. O procedimento de decapitação foi realizado em sala adjacente, a fim de evitar cheiro de sangue na sala de experimentos. A seguir, o cérebro foi dissecado e o hipotálamo removido por seção coronal de 2 mm de profundidade, distal à sutura de Bregma (2 a 4mm), estendendo-se da decussação supra-óptica até o término dorsal da parte inferior do terceiro ventrículo, em no máximo quatro minutos. O hipotálamo foi imediatamente colocado em nitrogênio líquido e armazenado a -80 °C. Sangue do tronco foi coletado em seringas com citrato de sódio a 4% e centrifugado a 900G, 4 ^{o}C, por 20 minutos. O plasma foi então armazenado a -20°C para medidas de glicose posterior.

Ensaios

A glicose plasmática foi medida em duplicata pelo método da glicose oxidase utilizando os estojos comerciais do CENTERLAB (Santa Luzia, Minas Gerais, Brazil) como um parâmetro para confirmar o estresse dos animais, e para comparar os procedimentos de estresse com relação a suas respostas glicêmicas.

A AMPQ foi avaliada em condições basais e após 20 minutos de cada estresse. A escolha para avaliação de AMPQ após 20 min de cada estresse baseou-se em nossa experiência prévia e de outros mostrando um pico de resposta hiperglicêmica e de hormônios de estresse em torno de 20 minutos [41-44]. Ensaios de imunoprecipitação para AMPQ, usando anticorpos anti-peptídeo, foram previamente descritos[45]. Em suma, as amostras de tecidos foram homogenizadas e o conteúdo protéico foi determinado (BCA assay, Pierce, Rockford, USA). A atividade de AMPQ no complexo imune foi determinado por fosforilação de SAMS – um peptídeo sintético que serve como substrato para a AMPQ. 300g de proteína foram imunoprecipitados usando esferas de proteína G (Amersham Biosciences, Bucks, UK) e uma mistura de 1 e 2-alfa anticorpos AMPQ (2.5 g/amostra de cada). O imunoprecipitado foi dividido em três alíquotas, e dois foram usados para medida da atividade de AMPQ com uma solução de reação contendo 0.1 Ci de [32P] ATP, 0.1l of 100 mM de ATP não irradiante, 0.25l de 1M $MgCl_2$, 1 l de 1mM AMP, e 10l de SAMS 0.1mM (Upsate Biotechnology, Dundee, Scotland) e a terceira alíquota com a mesma mistura exceto SAMS (substituído por Hepes-Brij). A reação foi realizada em um agitador a 30°C por 20 minutos, e as amostras foram pipetadas em quadrados de papel (P81; Upstate Biotechnology).

A reação foi parada pela colocação dos quadrados de papel em gelo e em ácido fosfórico a 1%. A atividade radioativa foi contada usando um contador cintilográfico.

Para medidas da atividade de AMPQ 1 e 2, 600g de proteína foram usadas e imunoprecipitação foi realizada seqüencialmente para AMPQ 1 e 2. A atividade da AMPQ foi calculada usando-se a diferença de contagens entre as amostras contendo SAMS e as amostras não contendo SAMS (SAMS negativas) e foi expressa em nanomoles de ATP incorporado por minuto por miligrama de amostra de peptídeo. A atividade de AMPQ (α1 + α2) foi apresentada em valores de porcentagem a fim de corrigir o decaimento natural diário da radioatividade do ATP radioativo.

Análise Estatística

Verificou-se distribuição Gaussiana dos dados por dois diferentes testes – D'Agostino-Pearson e Shapiro-Willk. A análise da ingestão hídrica e ganho de peso foi realizada por teste T de Student não pareado. Os dados de glicose plasmática foram analisados por ANOVA seguido por Bonferroni. *Os dados de* AMPQ foram testados por Kruskal-Wallis seguido por comparação de Conover-Inman. O GraphPad Prism 4 (São Francisco, CA, USA) foi utilizado para análise estatística. Todos os dados foram expressos como média ± erro-padrão (EP), e significância foi considerada como $p < 0.05$.

Resultado

Ingestão Hídrica e Ganho de Peso

A ingestão diária de água e o ganho de peso de ratos cronicamente tratados com candesartan por duas semanas foram comparados com os controles. Não houve diferença significativa entre a ingestão diária de água com candesartan e de água sem adição dos controles (21.6 ± 1.0 *versus* 19.5 ± 1.3 ml/100g/dia respectivamente, $p>0.05$).

O peso inicial dos ratos tratados com candesartan não diferiu significativamente dos controles (211.3 ± 4.0 *versus* 211.3 ± 3.5g respectivamente, $p>0.05$).

Níveis de Glicose Plasmática

Como mostrado na figura 1 para estresse de contenção (protocolo 2), não há diferença significativa nos níveis de glicose plasmática em ratos não estressados tratados com candesartan quando comparados com controles não estressados (87.5 ± 4.2 *versus* 88.9 ± 1.7 mg/dl respectivamente, $p>0.05$). Após 20 minutos de contenção, os níveis plasmáticos de glicose aumentaram significativamente em ambos os grupos tratados com candesartan (125.8 ± 5.1 *versus* 87.5 ± 4.2 mg/dl, $p < 0.05$), e controles (126.0 ± 3.2 *versus* 88.9 ± 1.7 mg/dl, $p<0.01$). Todavia, ausência de diferença significativa entre os grupos pode estar relacionada ao tratamento com candesartan.

Da mesma forma, no estresse cirúrgico (protocolo 3) também mostrado na figura 1, os níveis basais de glicose plasmática para o grupo tratado com candesartan não foi significativamente diferente quando comparado ao controle (83.8 ± 5.3 *versus* 86.9 ± 4.4 mg/dl, $p >0.05$). Após 20 minutos de estresse cirúrgico, os níveis de glicose plasmática aumentaram significativamente em ambos os grupos tratados com

candesartan (163.8 ± 11.1 *versus* 83.8 ± 5.3 mg/dl, p < 0.01), e controles (165.0 ± 14.7 *versus* 86.9 ± 4.4 mg/dl, p<0.01). Ausência de diferença significativa entre os grupos pode ser atribuída ao tratamento com candesartan. De maneira interessante, as respostas hiperglicêmicas associadas aos diferentes estresses foram diferentes também. A contenção evocou uma menor resposta hiperglicêmica ao estresse do que o estresse cirúrgico sob anestesia em ambos os animais tratados com candesartan e os controles (125.8 ± 5.1 *versus* 163.8 ± 11.1 e 126.0 ± 3.2 *versus* 165.0 ± 14.7 mg/dl respectivamente, p<0.01 para ambas as comparações), confirmando nosso relato prévio[18].

Atividade de AMPQ Hipotalâmica

Como mostrado na Figura 2, houve um aumento da atividade hipotalâmica basal nos ratos tratados com candesartan quando comparados com controles (163.5 ± 17.7% *versus* 100.0 ± 11.4% do controle, p < 0.01). Após 20 minutos de contenção, a atividade de AMPQ hipotalâmica aumentou significativamente no hipotálamo de controles estressados quando comparados com controles não estressados (180.3 ± 18.3% *versus* 100.0 ± 11.4%, p<0.01), correspondendo a um aumento de aproximadamente 80% se comparado aos valores basais. Por outro lado, a atividade da AMPQ não aumentou em animais tratados com candesartan após 20 minutos de contenção se comparado com os respectivos valores basais (145.6 ± 9.5% *versus* 163.5 ± 17.7%, p >0.05). Estes valores continuaram mais elevados do que os controles não estressados (145.6 ± 9.5% *versus* 100.0 ± 11.4% do controle, p < 0.05).

No protocolo 3 (Figura 2), foi também observado um aumento da atividade da AMPQ hipotalâmica em ratos tratados com candesartan quando comparados com os controles (158.0 ± 22.3% *versus* 100.0 ± 10.2% de controle, p < 0.05). Após 20 minutos de estresse cirúrgico sob anestesia geral, nenhuma diferença significativa pode ser observada na AMPQ hipotalâmica dos controles quando comparado com seus valores basais (95.4 ± 12.1% *versus* 100.0 ± 10.2% do controle, p > 0.05), enquanto uma redução significativa da atividade da AMPQ foi detectada no grupo estressado tratado com candesartan quando comparado com os valores basais respectivos (81.1 ± 12.0% *versus* 158.0 ± 22.3%, p < 0.01).

Os resultados deste estudo demonstraram que: 1) a atividade de AMPQ hipotalâmica é aumentada pela administração crônica de candesartan em animais não estressados. 2) a atividade da AMPQ hipotalâmica é aumentada por contenção, mas não é alterada por estresse cirúrgico sob anestesia geral. 3) o tratamento crônico com candesartan previne aumentos posteriores na atividade de AMPQ durante contenção, e reduz a atividade da AMPQ no estresse cirúrgico sob anestesia geral a valores observados em controles não estressados.

F

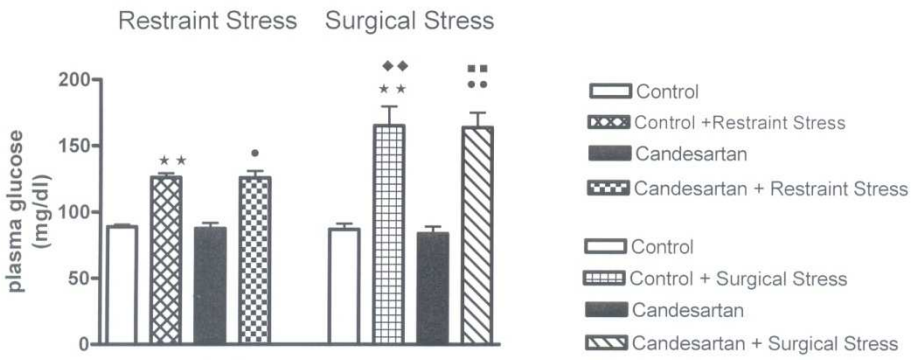
Restraint Stress
Surgical Stress
plasma glucose (mg/dl)
200
150
100
50
0
Control
Control +Restraint Stress
Candesartan
Candesartan + Restraint Stress
Control
Control + Surgical Stress
Candesartan
Candesartan + Surgical Stress

Figura 1

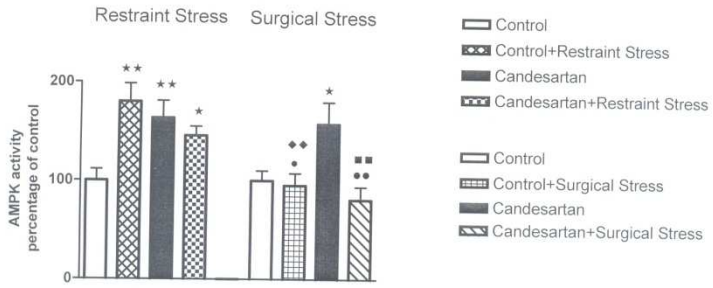
Restraint Stress
Surgical Stress
AMPK activity
percentage of control
200
100
0
Control
Control+Restraint Stress
Candesartan
Candesartan+Restraint Stress
Control
Control+Surgical Stress
Candesartan
Candesartan+Surgical Stress

Figura 2

Legendas das Figuras

Figura 1: Efeitos dos Estresses de Contenção e Cirúrgico Sobre os Níveis de Glicose Plasmatica em Ratos Cronicamente Tratados com Candesartan. Valores expressos como medias ± EP (erro-padrao).

** p < 0.01 para comparação com Controles; • p < 0.05 para comparação com Candesartan

e •• p < 0.01 para comparação com Candesartan; ♦♦ p < 0.01 para comparação com Controle + Estresse de contenção; ▪▪ p < 0.01 para comparação com Candesartan + Estresse de contenção.

Figura 2: Atividade da AMPQ Hipotalâmica Basal e Após Estresse de Contenção em Ratos Cronicamente Tratados com Candesartan. Valores expressos como media ± EP (erro-padrao).

* p < 0.05 para comparação com controle; ** p < 0.01 para comparação com controle

Figura 2: Atividade da AMPQ hipotalâmica basal e após estresse cirúrgico em ratos cronicamente tratados com Candesartan. Valores expressos em medias ± EP (erro-padrao).

* p < 0.05 para comparação com controle; • p < 0.05 para comparação com Candesartan e

•• p < 0.01 para comparação com o Candesartan.

Discussão

A AMPQ é um sensor metabólico regulador com inúmeros substratos-alvo envolvidos no metabolismo de proteínas, carboidratos e lípides, e na utilização de energia. A AMPQ não é um sensor apenas do *status* energético, mas também pode ser ativada por hormônios (como a leptina) e estresses (como o estresse osmótico) que não alteram a relação AMP/ATP. Além disto, estímulos fisiológicos e patológicos podem ativar a AMPQ, como exercício físico, jejum, hipóxia, hipoglicemia e isquemia[22]. O cérebro é o órgão-chave do estresse, sensor e efetor. A AMPQ expressa-se em todo o cérebro e especialmente no hipotálamo, onde funciona como um sensor energético para todo o organismo[46]. Até 2006, os bloqueadores de receptores da angiotensina II que reconhecidamente tinham atividade PPAR gama agonista eram telmisartan e ibersatan[47]. Depois, foram publicados estudos mostrando que o candesartan também é capaz de aumentar a atividade PPAR gama, capaz de aumentar a AMPQ ativada[48].

Este estudo é o primeiro a descrever a modulação do candesartan sobre a AMPQ hipotalâmica na ausência de estresse e nos estresses de contenção e cirúrgico. Os bloqueadores de AT1 são extensamente empregados na prática médica e pouco se sabe sobre os seus efeitos no sistema AMPQ. Nosso experimento demonstra que o candesartan pode alterar significativamente a atividade basal da AMPQ hipotalâmica assim como após contenção ou estresse cirúrgico sob anestesia geral, interagindo de forma complexa.

Os resultados deste estudo demonstram que: (1) a atividade hipotalâmica é aumentada por contenção, mas não é alterada por estresse cirúrgico sob anestesia geral; (2) a atividade da AMPQ hipotalâmica é aumentada pela administração crônica de candesartan a animais não estressados; e (3) o tratamento crônico com candesartan previne aumentos de AMPQ ativada subseqüentes na contenção e reduz a atividade da AMPQ no estresse cirúrgico sob anestesia geral a valores observados em controles não estressados.

Em adição ao seu papel terapêutico na regulação da pressão arterial e função vascular, os bloqueadores AT1 parecem melhorar a regulação metabólica, embora os mecanismos de ação ainda tenham de ser melhor esclarecidos[49,50]. Recentemente demonstrou-se que o candesartan aumenta a expressão do receptor nuclear PPAR gama e da adiponectina[48], ambos capazes de aumentar diretamente a atividade da AMPQ hipotalâmica[51]. Tanto o agonismo do receptor PPAR gama quanto o aumento de adiponectina podem ter contribuído para o aumento observado da atividade de AMPQ hipotalâmica basal verificado em nosso estudo[47,48,52].

O efeito anorexigênico do bloqueador de receptor AT1 com propriedades agonistas PPAR gama foi recentemente descrito[53]. No nosso experimento, embora não desenhado especificamente para avaliar a regulação de apetite, observamos um reduzido ganho de peso em animais tratados com candesartan. Também observamos que o tratamento com candesartan aumenta a atividade basal da AMPQ no fígado (dados não publicados), o que está de acordo com acordo a atividade agonista periférica PPAR gama e seus efeitos benéficos no metabolismo. Alternativamente, a bem descrita indução de óxido nítrico sintetase endotelial (e-NOS) com aumentada bioviabilidade do óxido nítrico e sua conseqüente proteção contra isquemia cerebral [54,55] também podem estar associadas a este aumento de AMPQ em animais não estressados tratados com candesartan[56]. Acreditamos ser de grande importância verificar se a administração pré-operatória de candesartan e níveis de AMPQ hipotalâmica elevados pode ser neuroprotetora em cirurgias de clipagem temporária de artéria cerebral, como as de clipagem de aneurisma, correção de má formação arteriovenosa, e endarterectomia carotídea.

As diferentes respostas hiperglicêmicas aos estresse de contenção e cirúrgico foram reproduzidas neste estudo, corroborando o conceito de acentuada heterogeneidade das respostas neuroendócrinas ao estresse com sua ativação específica de circuitos centrais e assinatura neuroquímica própria, como descrito por outros autores[52,57-59]. Da mesma forma, a atividade da AMPQ hipotalâmica em nosso estudo foi demonstrada como respondendo diferencialmente aos vários estressores. Após contenção, os animais do grupo controle aumentaram sua atividade de AMPQ hipotalâmica, enquanto nenhuma alteração da atividade de AMPQ hipotalâmica foi demonstrada após cirurgia sob anestesia geral. De fato, a contenção aguda tipicamente ativa os neurônios dos núcleos paraventriculares do hipotálamo[60], e estes núcleos estão intimamente relacionados com a atividade hipotalâmica da AMPQ[61]. Por outro lado, a anestesia geral parece prevenir o excesso de consumo energético e, desta forma, teoricamente poupar a AMPQ sob estresse cirúrgico. O α2 adrenoreceptor agonista xilazina utilizado neste experimento age centralmente para diminuir a atividade simpática eferente e as concentrações de norepinefrina circulantes preservando assim o consumo de energia. Recentemente, demonstrou-se que quetamina, um receptor antagonista de N-metil-D-ácido aspártico (NMDA), reduz a atividade da AMPQ cerebral quando administrada a ratos antes de isquemia. Portanto, a combinação de quetamina e xilazina pode ter prevenido o aumento AMPQ após estresse cirúrgico em animais do grupo controle anestesiados e este bloqueio pode ter sido potencializado pela resposta hiperglicêmica mais acentuada em resposta ao estresse cirúrgico[61].

Conclusão

O papel da angiotensina II na regulação das respostas ao estresse foi previamente investigado[63-65]. De forma interessante, foi demonstrado que o estresse de contenção agudo aumenta o RNAm dos receptores AT1 nos núcleos paraventriculares[66] enquanto a administração periférica crônica de candesartan bloqueia os receptores AT1 cerebrais[67-69], inibindo as respostas centrais à angiotensina II em ratos[52,69,70]. Além disto, o candesartan foi recentemente descrito como um composto anti-estresse[71], melhorando as desordens relacionadas ao estresse e reduzindo as respostas do cortisol à estimulação do hormônio liberador de corticotrofina[72]. Portanto, o bloqueio de receptores AT1 dos núcleos paraventriculares provavelmente relaciona-se a ausência de aumentos posteriores da atividade da AMPQ hipotalâmica em animais tratados com candesartan submetidos a contenção. Além disto, o efeito anti-estresse global do candesartan associado aos efeitos da anestesia acima mencionados reduziu a atividade da AMPQ hipotalâmica em animais tratados com candesartan submetidos a estresse cirúrgico.

Curiosamente, embora a atividade da AMPQ hipotalâmica esteja aumentada em animais tratados com candesartan não estressados, o tratamento com os bloqueadores de receptor AT1 não interfere com o efeito da anestesia na atividade da AMPQ hipotalâmica. Assim, em vigência de insulto cerebral intraoperatório, quando a anestesia é protetora e poupa AMPQ, o tratamento pré-operatório com candesartan não bloqueia este efeito.

Apesar de sua originalidade, este estudo não foi desenhado para elucidar os mecanismos moleculares subjacentes, o que constitui sua principal limitação. Nossos dados demonstram um quadro *in vivo* da variabilidade de respostas a estressores comuns e o papel abrangente da AMPQ em estresse e anestesia. Seguindo políticas para o uso do número mínimo necessário de animais em experimentos, nós não incluímos um grupo de ratos submetidos a anestesia separadamente (sem cirurgia) uma vez que este cenário não é relevante para situações clínicas. Estudos futuros possivelmente podem demonstrar as vias moleculares envolvidas nas alterações de AMPQ seguindo estes estressores comuns.

Em suma, este estudo *in vivo* mostra que o bloqueador de receptor AT1 da angiotensina II candesartan altera diferentemente a atividade hipotalâmica de AMPQ, dependendo se em situação basal (sem estresse) ou após um evento estressante agudo. A AMPQ é ativada na ausência de estresse pelo candesartan, mas a AMPQ é reduzida após os estresses de contenção e cirúrgico, o que pode ser atribuído às propriedades anti-estresse do candesartan. Além disto, a AMPQ hipotalâmica parece responder de forma única a cada estresse e foi demonstrado que a combinação de quetamina e xilazina reduz a AMPQ hipotalâmica quando associada ao candesartan no estresse cirúrgico. Estudos futuros devem investigar a importância da associação de candesartan à anestesia para prevenir dano neurológico intraoperatório através de alteração da AMPQ hipotalâmica, especialmente no cenário clínico de pacientes com síndrome metabólica.

Nós concluímos, portanto que o entendimento da conexão entre o sistema AMPQ e os bloqueadores de receptors AT1 da angiotensina II e sua modulação em procedimentos estressantes pode constituir-se em alvo de novos tratamentos na moderna prática anestesiológica.

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ANEXOS

1) Cerebral Activation of the AMP-activated protein kinase through the lens of Stressor-specificity and Allostatic Load Concepts (REVIEW)

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The AMP-activated protein kinase (AMPK) is a heterotrimetric kinase of the serine-threonine family present in all eukaryotic cells and conserved throughout evolution. AMPK plays a prominent role as a metabolic and stress sensor. Accordingly, AMPK is activated under circumstances of ATP and glycogen depletion, and its activation leads to inhibition of ATP-consuming anabolic pathways, assuring survival upon strain. AMPK activation may assume peculiar roles according to the magnitude of the threatening, stressor specificity and the organ. In the brain; hypoxia, hypoglycaemia, stroke/ischaemia, fasting, and reactive species of oxygen have been associated with AMPK activation. Moreover, activation of AMPK in the hypothalamus will lead to increased appetite, which represents a survival mechanism during the species evolution. By contrast, over-activation of cerebral AMPK represents allostatic load and may lead to neuronal damage. In the heart, fuel is selected by increases on AMPK activity and depends on the stressor. During ischaemia, glycolysis increment is associated with insulin-regulated glucose transporter translocation to cardiomyocyte membrane promoted by AMPK. On the other hand, during reperfusion, enhanced fatty acid oxidation is the overall consequence of Acetyl-CoA carboxylase phosphorylation. We review the role of AMPK activation through the lens of stressor-specificity concept and in an organ-dependent manner, speculating whether it can be protective or harmful.

Key-words: AMPK, stress, stressor-specificity, ischaemia, allostatic load, homeostasis.

Content:

1- AMPK Concept

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme from the serine/threonine family, highly conserved throughout evolution[1,2]. Structurally, AMPK comprises the catalytic α-subunit, the scaffold β-subunit and the regulatory γ-subunit. It is found in every eukaryotic cell, from yeast to plants, and to mammals. AMPK is expressed in most mammalian tissues and participates in regulation of fuel supply in response to metabolic increasing demands of liver, central nervous system, fat tissue, skeletal muscles, and myocardium [3].

AMPK functions as a metabolic and stress sensor that assures survival during energy-depleting processes. AMPK is frequently compared to a fuel gauge that senses in the cell the depletion of ATP and glycogen, and the increasing AMP levels, thus activating energy-producing pathways. In fact, AMPK system responds in an ultra sensitive manner over a critical range of AMP concentrations, in that only a 6-fold increase of nucleotide is required for the maximal activity of the kinase to progress from 10% to 90%[4].

AMPK directly controls metabolic enzymes, activates transcription, and mediates the metabolic effects of hormones such as glucocorticoids, insulin, leptin, ghrelin, adiponectin, and cannabinoids[5]. AMPK also plays an important role in different types of stress such as hypoxia, ischaemia and hypoglycaemia. The evolution of the stress concept will be further discussed in this chapter.

In the recent years, modulation of AMPK activity has been the focus of numerous studies. This can be explained by the fact that G-protein coupled receptors (GPCRs), that comprise the largest family of membrane receptors in the human genome, have been shown to mediate part of their effects through activation of AMPK[6]. GPCRs are target for approximately 30% of all current therapeutic agents. Furthermore, there are other targets of AMPK besides regulator of metabolic pathways that have continuously being described such as cell growth, cytoskeletal organization, and cell cycle

regulation. Whether AMPK activation can be protective or harmful is of outstanding concern to diabetes[7], obesity[8], stroke[9], ischaemic heart disease[10] and cancer therapeutics [11,12].

1.1- History and Relevance

AMPK was discovered over 3 decades ago as a protein kinase associated with acetyl-CoA carboxylase (ACC1)[13] and HMG CoA reductase (HMG-CoA)[14], which are key regulatory enzymes for fatty acid and cholesterol synthesis. Subsequently, it was found to be activated by AMP and it was then recognized that AMPK was itself regulated by cellular energy levels in the form of AMP or phosphorylation [15,16].

The importance of AMPK in the cell has been emphasized by the discovery that it is responsible for the phosphorylation of numerous proteins involving disparate cellular functions, and that mediates the actions of type 2 diabetic drugs[16]. Furthermore, AMPK might mediate many of the health benefits of physical exercise in mitigating obesity, insulin resistance and type 2 diabetes. Altogether, AMPK is now recognized as a multifunctional metabolic cellular sensor as well as a stress sensor that has been functionally conserved throughout eukaryotic evolution[1].

The activation of the AMPK system by various physiological and pathological stimuli such as ischaemia[17], hypoxia[18], exercise[7], starvation[19], metabolic poison (arsenite, olygomicin)[20], oxidative stress[21], low glucose[22], and osmotic stress[23], promotes energy restorative processes while inhibiting energy consumptive processes.

1.2- Structure and Regulation

AMPK is structurally a αβγ heterotrimer (Figure 1) which belongs to the serine/threonine family.

The catalytic α subunit contains a conventional Ser/Thr kinase domain, followed by an autoinhibitory sequence and a C-terminal segment for interacting with the β-subunit [24,25]. The scaffold β subunit bridges α- and γ-subunits by means of its C-terminal sequence, and contains a central non-catalytic glycogen-binding domain that may sense the status of cellular energy reserved in the form of glycogen[26, 27]. The regulatory role of the β subunit in modulating AMPK activity has been highlighted in studies of the thienopyridone A-769662 that directly activates AMPK by an unexpected mechanism[28,29] involving the beta subunit carbohydrate-binding module and residues from the γ subunit but not the AMP binding sites. The regulatory γ subunit is the site for AMP binding that may ultimately remove the effect of the auto-inhibitory domain (AID) on kinase activation and pThr172 dephosphorylation[30].

AMPK is activated by AMP both allosterically and by inhibiting dephosphorylation[14, 31]. Furthermore, AMPK is activated through phosphorylation by 3 upstream kinases, the tumor suppressor LKB1 complex, calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ), and the transforming growth factor-β-activated kinase 1 (TAK1)[32,33,34]

AMPK transfers high-energy phosphate from ATP to target proteins, such as for e.g. ACC1 and HMG-CoA which are inhibited as a consequence of this phophorylation. AMPK has also been reported to activate endothelial nitric oxide synthase (eNOS),

thereby controlling nitric oxide (NO) bioavailability which is ultimately crucial in cardiovascular homeostasis.

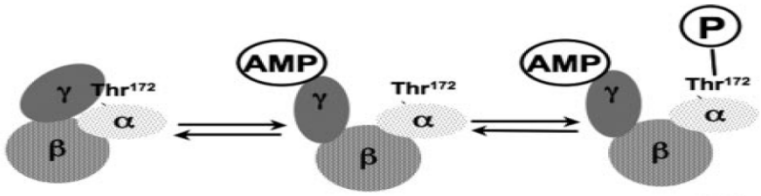

Fig 1. Activation of the AMPK system.

2- Stress Concept

Stress is a challenging or threatening experience that elicits physiological or emotional responses with the purpose of sustaining homeostasis and assuring exhilaration, accomplishment, and indeed, survival.

A hallmark of the stress response is the activation of the autonomic nervous system and of the hypothalamus-pituitary-adrenal (HPA) axis, and a given organism depends upon normal stress hormone responses for proper survival.

2.1- Evolution of the Stress Concept

Stress concept has evolved during the last century and is in continuous change. The main stress theories and their respective authors are related in Table 1.

In 1929, Cannon introduced the term 'homeostasis' to describe the coordinated physiological processes which maintain most of the steady states in the organism[35]. He focused on the sympathetic nervous system as essential to restore homeostasis and to promote survival of a stressed organism. Cannon also demonstrated that the specific homeostatic reaction to lack of oxygen is quite different from that required to resist to a cold exposure. He properly stated that a stereotyped response pattern would not have provided a clear advantage in natural selection[35-37].

Selye first introduced the term 'stress' as a medical concept. In a yet to become a historical letter to Nature, in 1936, Selye described adrenal enlargement, gastrointestinal ulceration and thymicolymphatic involution as a pathological triad produced by a variety of noxious agents[38]. From this starting point, he described the general adaptation syndrome evolving as a sequence of alarm, resistance and exhaustion stages. Later, Selye proposed that a common stress response could follow all stressors and involve the release of ACTH and adrenal corticosterone. Furthermore, it was proposed that individual stress hormone responses could be mediated by "conditioning factors" such as genetically determined predispositions[39,40]. Several decades later, Mason and others proposed a re-evaluation of the concept of "non-specificity" in stress theory, showing that activity of the HPA axis could increase, decrease, or remain unchanged in response to different stressors[41].

Thus, the concept of a non-specific, general, stereotyped acute stress response was opposed to the idea that each stressor evokes its own particular specific neuroendocrine response. However, the "flight-or-fight" response is the classical way of envisioning the behavioral and physiological response to an acute stress. On the other hand, the stressor-specific response with its specific central-activated circuit and neurochemical signature has challenged the concept. Nevertheless, there are many current views concerning stress definition although none has been widely accepted.

McEwen reported hippocampus rat brain binding corticosteroids [42,43]. Subsequently, he described chronic stress as related to remodeling of neurons [44,45] – a process called 'neuroplasticity'. Weiner[46], Chrousos and Gold[47], and Goldstein[48] have also made important contributions to the field, and they have put stress as a source of autoimmune, inflammatory and cardiovascular diseases among others (Table 1).

Recently, another mainstream theory was suggested by molecular biologists regarding the role of heat shock proteins in cellular survival[49]. It has been reported that the cell always reacts in the same way to strain, regardless of the insult. By contrast, Gaillet[50], and Pácak and Palkovits[51-53] have demonstrated through numerous studies a marked heterogeneity of neuroendocrine responses to various stressors. They have already mapped cerebral stressor-specific pathways and circuits, using intracerebral microdialysis and immunohistochemistry. Nevertheless, a worldwide definition of stress is still a matter of debate.

Table 1. Stress theories

Author	Contribution to the stress field	Reference
Bernard	*The term* Milieu interieur was first introduced.	(75)
Cannon	The term homeostasis was first introduced.	(35)
Selye	The terms stress, eustress, distress, and stressor were introduced. Stress was defined as the body nonspecific response to any threaten (general adaptation syndrome).	(38-40)
Mason	Selye´s doctrine of nonspecificity was criticized. Anxiety and fear were considered as the main factors contributing to nonspecific responses upon exposure to various stressors.	(41)
Hennesy and Levine	Introduced a "psychoendocrine hypothesis" of stress and arousal.	(76)
Krantz and Lazar	Defined psychological stress as a "transaction" between an organism and its environment.	(77)
Munck and Guyre	Incorporated inhibitory effects of glucocorticoids to the development of "diseases of adaptation".	(78)

Levine and Ursin	Incorporated adaptive biological responses into the definition of stress.	(58)
Weiner	*Stress was defined as* an external experience or phenomena to the organism.	(46)
Chrousos and Gold	Stress was defined as a state of disharmony or of threatened homeostasis evoking both specific and nonspecific responses. Genetic polymorphisms were included as important determinants of individual stress responses.	(47)
Goldstein	Stress was defined as a condition where expectations, whether genetically programmed, established by prior learning, or deduced by from circumstances, do not match the current or anticipated perceptions of the internal or external environment. The discrepancy between what is sensed and what is expected was described as eliciting patterned, compensatory responses.	(48)
McEwen	Incorporated the term allostasis and allostatic load as the active process of adaptation of the body upon the exposure to various stressors. Receptors for adrenal steroids were demonstrated in the hippocampal formation. Remodeling of neurons was shown to be related to restrain and chronic stress evoking disease states.	(42-45)
Pacák and Palkovits	Observed that each stressor-specific response has its own neurochemical signature.	(51-53)

2.2- Stressful Stimulus and Its Classification

According to duration, stressors can be classified as acute (single, intermittent, and time-limited exposure versus continuous exposure) or chronic (intermittent and prolonged exposure versus continuous exposure).

During an acute stress response, physiological processes are important to redirect energy utilization among various organs and selectively inhibit or stimulate organ systems to mobilize energy reserve. Thus, certain tissues reduce their consumption of energy while those important for locomotor activity receive sufficient nutrients from the circulation as well as the central nervous system.

Stressors can be divided into four main categories: physical stressors with a negative or positive psychological component; psychological stressors that reflect a learned response to previously experienced adverse conditions; social stressors reflecting disturbed interactions among individuals; and stressors that challenge cardiovascular and metabolic homeostasis. Examples of physical stressors are cold, heat, radiation,

noise, vibration, and others. Psychological stressors refer to those affecting emotional processes and may result in behavioral changes such as anxiety, fear, and frustration. Social stressor consists otherwise on placement of an animal into the territory of a dominant animal. In humans, it could be the consequence of mourning, divorce and unemployment. Stressors disturbing cardiovascular or metabolic homeostasis typically include severe exercise, heat exposure, hypoglycaemia, hemorrhage, etc. Handling and immobilization are also good examples of mixed stressors especially used in animal research.

2.3- Allostasis and Allostatic Load

The term allostasis was introduced by Sterling and Eyer[54] and literally means "achieving stability through change". It refers to the active process by which the body responds to daily events and maintain homeostasis. For example, the body responds to simple acts like getting out of bed in the morning by releasing cathecolamines that increase heart rate and blood pressure. In this sense, allostasis produces protection. Moreover, allostasis means the process of maintaining stability through the active release of stress hormones and various mediators such as adrenal steroids, cathecolamines, cytokines, and tissue mediators [44].

On the other hand, chronic elevation of the same mediators can lead to pathophysiological changes such as arterial hypertension and atherosclerosis, and result in stroke, myocardial infarction and renal insufficiency. In this very way, over time, chronically increased allostasis can induce damage. Considering the paradoxical actions of these mediators, the term allostatic load or overload was introduced to refer to the wear and tear on the brain and body when mediators are dysregulated, i.e., turned on when stress is over or turned off when they are needed, [55,56].

2.4- Clinical Relevance of Stressor Specificity

Mason properly observed that the activity of the HPA axis could increase, decrease or remain unchanged in response to different stressors[41]. Moreover, threatened homeostasis evokes an adaptive compensatory specific response of the organism. The adaptive response is characterized by activation of specific central circuits genetically programmed and modified by the environment.

The role of neuroendocrine responses in coping with stress is well recognized and these responses have permitted survival of a given organism to many stressful situations[57]. Thus, physiological systems must be turned on efficiently by a particular stressor and turned off when a stressor has ceased[58]. When the neuroendocrine systems are not efficiently mobilized and then appropriately reduced, elevated hormone levels result in stress-related diseases e.g., hypertension, diabetes, stroke, obesity, autoimmune and inflammatory diseases, gastritis and others .

2.5- The brain as a target of stress

The brain is the key organ of the stress response as it both recognizes threatening and elaborates the physiological and behavioral responses which can be either adaptive or

damaging[52]. Stress involves two-way communication between the brain and the cardiovascular, immune, and other systems via neural and endocrine mechanisms.

Beyond the classical 'flight-or-fight' response to acute stress, there is activation of stressor-specific pathways. Hormones associated with stress protect the body in the short-run and promote adaptation – allostasis. However, stress and its hormones produce both adaptive and maladaptive effects. Throughout the daily life, chronic stress acts on these brain regions wearing and tearing on the body – allostatic load. The hippocampus, amygdala, and prefrontal cortex undergo stress-induced structural remodeling.

Threat to homeostasis or stress of almost any kind will cause plasma glucocorticoid levels to rise. The increased levels have been traditionally been ascribed the physiological function of enhancing the organism's resistance to stress. The hypothalamus and the hippocampus were the first recognized target of glucocorticoids [42,43]. Besides glucocorticoids, excitatory amino acids in the hippocampus play a prominent role in the aging process and to the damage that can result from the severe stress of ischaemia and seizures[58,59].

3- AMPK as a Stress Sensor in the Brain

The brain is one of the organs with the highest energy consumption in the organism, and it uses glucose as the main substrate. The brain has a low storage energy capacity and relies on allocation of energy resources between itself and the periphery, or intake of nutrients[60].

Considering that the brain gives priority to regulate its own adenosine triphosphate (ATP) concentration and is sensitive to cellular energy fluctuations, it is expected that AMPK plays an important role on the survival of neurons [61,62].

AMPK plays a central role in appetite regulation and this role of AMPK in hypothalamic neurons integrating nutritional and hormonal signals (ghrelin, leptin and adiponectin) has been recently reviewed [63-64]

3.1- Neuronal effects of AMPK

In a study of rat cultured neurons from hippocampus, it was demonstrated that AICAR promoted survival of neurons exposed to glucose deprivation, hypoxia and glutamate and amyloid beta-peptide. The protective effect of AICAR was AMPK mediated as suppression of levels of the AMPK alpha1 and alpha2 subunits resulted in enhanced neuronal death following glucose deprivation, and abolished the neuroprotective effect of AICAR[62].

In rats submitted to transient occlusion of the middle cerebral artery, it was observed significant increase in AMPK activity. As a consequence, phosphorylation of the $GABA_B$ receptors is enhanced. $GABA_B$ receptors are important either for the induction of slow and prolonged synaptic inhibition in the brain[65], and also reduces excitotoxicity and promotes neuronal survival.[66]

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Resveratrol, a plant-derived phenol compound that protects against global cerebral ischemic injuries [9,67], has interestingly been shown to activate AMPK in cultured neurons and in the mouse brain[68]. Some of the beneficial actions of resveratrol, including enhanced mitochondrial biogenesis, are linked with the AMPK system. After hypoxic-ischemic brain injury, fast-enhanced mitochondrial biosynthesis could hypothetically improve the oxidative state of the brain and induce neuronal repair [69].

On the other hand, there are studies suggesting that over-activation of AMPK representing allostatic load can have a negative impact on neuronal protection. McCullough et al[70] demonstrated reduced stroke damage after pharmacological AMPK inhibition through compound C and C75. Furthermore, knockout of AMPK α2-subunit elicited a protective effect in the same stroke model[71]. It was suggested that the two mammalian AMPK α-isoforms show different functions. Curiously, the α1-isoform locates primarily to the cytoplasm, while the α2-isoform locates to the nucleus and could play a role in transcription[72]. However, the precise mechanism by which subcellular localization of the subunit could modify the response to a certain type of stress such as the ischaemic injury remains to be determined.

The kainic acid is a potent agonist of glutamate receptors and has been shown to promote CaMKKβ-mediated activation of AMPK in glioma cells and in murine hipoccampus[73,74]. Yoon et al[74] showed that AMPK activation led to increased expression of brain-derived neurotrophic factor (BDNF), whose over-expression aggravates epileptic seizures in animal models. The role of AMPK-regulated BDNF expression during glutamate-induced epileptic seizure is yet to be determined.

In order to better define the role of AMPK in neuronal survival, other more specific activators and inhibitors shall be used before a definitive conclusion could be draw.

4- Conclusions

Recognized as being associated with ACC1 and HMG-CoA for decades, AMPK has emerged as an extremely important metabolic and stress sensor, to be the target of anti-diabetic and anti-cancer drugs. However, the aforementioned data raise intriguing questions about the role of cerebral AMPK in different stress models.

The specificity of each stressor with its own neurochemical signature turns on the possibility of different AMPK responses according to stress type and analysed organ. Further AMPK *in vivo* studies are therefore necessary to establish whether and in which conditions a sudden change in AMPK levels would be harmful or protective. Another important question is whether sublocalization of AMPK isoforms could affect the response to ischaemia among other stressors.

In the brain, on a model of transient occlusion of the middle cerebral artery, AMPK-mediated activation of the $GABA_B$ receptors reduced excitotoxicity and promoted neuronal survival. Care must be taken, however, if AMPK system is put under allostatic load. More *in vivo* studies are vital to assess the AMPK system performance under different stress models so as determining if AMPK's

activation is part of a survival response or represents wear and tear on the body.

Furthermore, the development of novel extremely specific AMPK activators and inhibitors is crucial to clarify the AMPK system role as a stress sensor, and could strengthen the evaluation of AMPK as a potential therapeutic target in different diseases such as diabetes, cancer, stroke, ischaemic heart diseases and heart failure.

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2) Effects of an angiotensin II receptor blocker on baseline hypothalamic AMP-activated protein kinase activity and after restraint and surgical stress in rats (Original Article).

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Running head: Hypothalamic AMPK differentially responds to candesartan at baseline and after stresses

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Brief Summary Statement: Candesartan augments baseline hypothalamic AMPK activity, but prevents the anxiety-induced AMPK rise and reduces AMPK following surgical stress under general anesthesia.

Abstract

Background: AMP-activated protein kinase (AMPK) has a prominent role as a metabolic stress sensor, having various roles according to the magnitude of the stressor specificity and the organ studied. AMPK is expressed in several areas of the brain, including the hypothalamus, and hypothalamic AMPK has been shown to play a role in stress and metabolism. Angiotensin II AT1 receptor blockers (ARBs) have been widely used in diabetes, a condition related to lower AMPK activity, but little is known about its relationship to AMPK either in basal conditions or during stressful events. This study aimed to evaluate hypothalamic AMPK at baseline and after restraint or surgical stress under ketamine and xylazine-induced general anesthesia.

Methods: Male Wistar rats were chronically treated with candesartan 5mg/kg/day in the drinking water for 2 weeks. After hypothalamus removal through decapitation, hypothalamic AMPK was determined at baseline and after restraint or surgical stress.

Results: We have found that hypothalamic AMPK activity is increased by chronic administration of candesartan in non-stressed animals ($p<0.05$); it is increased during restraint ($p <0.01$) but not influenced by surgical stress under anesthesia. Chronic treatment with candesartan prevents AMPK activity further increase in restraint, and decreases AMPK activity in surgical stress under anesthesia to values observed in non-stressed controls ($p<0.01$).

Conclusions: These modifications show that ARB may alter hypothalamic AMPK *per se*, and during both anxiety of restraint and surgical stress under anesthesia. Therefore, ARB seems to modulate hypothalamic AMPK, and this should be further explored to possibly improve surgical outcome.

Key words: AMPK, glucose, stress, candesartan, angiotensin receptor blocker, anesthesia

Introduction

AMP-activated protein kinase (AMPK) is a heterotrimeric kinase from the serine-threonine family present in all eukaryotic cells[1;2]. It plays a prominent role as a metabolic and stress sensor, mainly in terms of activation in response to an increasing AMP/ATP ratio, thereby improving survival under metabolic stress[3]. The activation of AMPK *in vivo* is complex and varies according to the magnitude and type of the stress as well as the organ studied[4]. The brain has been considered to be a key organ which needs to be protected during stress, and AMPK is highly expressed in several brain areas, especially the hypothalamus, where the role of AMPK has been investigated in some depth[5-7].

Angiotensin II AT1 receptor blockers (ARBs) have been considered as having a protective role in patients with diabetes, even when hypertension is not an issue[8;9]. In anesthetic practice, the number of patients chronically treated with (ARB) who are scheduled for surgery is increasing[10], and the role of these agents in perioperative medicine has not been fully determined. Moreover, it is known that AMPK can be also activated by certain drugs, hormones, and cellular stressors that do not alter the AMP/ATP ratio, such as osmotic changes. In addition, while AMPK has been extensively studied in terms of its response metabolic fuel deprivation[11], Very few studies are available on AMPK *in vivo* activation in common clinical scenarios, such as preoperative anxiety and during surgical procedures.

We therefore sought to evaluate hypothalamic AMPK activity under basal conditions and in response to extreme anxiety (restraint stress) or major surgery in anesthetized rats chronically treated with an ARB (candesartan) in order to obtain new insights in the role of the AMPK system in situations relevant to anesthesia.

Material and Methods

Animals

Male Wistar rats of 7-9 weeks of age (provided by the Federal University of Minas Gerais Medical School Animal Facility) were maintained under temperature-controlled conditions with an artificial 12-hour light-dark cycle, and allowed standard chow and water *ad libitum*. Rats were housed in individual cages and were daily handled. Animals were treated with candesartan cilexetil (AstraZeneca, Cotia, Brazil) 5mg/kg/day in the drinking water for 2 weeks (n=32) or no additions (vehicle) for the same period (n=32), according to a well established protocol[12;13]. The University Ethics Committee for Animal Experimentation approved all procedures (Protocol No. 110/2007).

Experimental design

Following the 2 week treatment with candesartan, rats were transferred in the early morning on the day of the experiment to the temperature-controlled experimental room and were left to rest for 60min in their home cages. The noise was kept at a minimum. Experiments were performed from 08.00h to 12.00h.

For the restraint stress, experiment animals were placed for 20min in a plastic polyethylene tube (21cm length, 4.5cm diameter), where they could not move, causing intense discomfort without apparent pain, for 20mins[14;15] . This experiment was used to mimic the typical anxiety related to preoperative period in clinical practice. For the surgical stress, experiment animals were anesthetized with 0.075mg/g intraperitoneal ketamine (Cristália, Itapira, Brazil) and 0.01mg/g xylazine (Schering-Plough Coopers, Cotia, Brazil) according to the protocol of the local Ethics Committee for Animal

Research. After an average of 5mins, when rats were deeply anesthetized, a median laparotomy was performed, followed by evisceration and exposure of the bowels for 20mins[15].

Non-stressed animals and those after 20mins of stress were moved to an adjacent room where they were killed by decapitation and the brain dissected. The hypothalamus was removed as a 2-mm thick coronal section from 2 to 4 mm from Bregma and from the supraoptic decussation to the dorsal end of the lower part of the third ventricle. The procedure took less than 3min. The hypothalamus was immediately placed in liquid nitrogen and stored at -80 °C. Blood from the trunk was collected in sodium citrate syringes and kept in ice until centrifugation (900G, 20min, 4□C). Plasma was then stored for subsequent glucose measurements.

Assays

Plasma glucose was assayed in duplicate by glucose oxidase utilizing commercial kits from CENTERLAB (Santa Luzia, Minas Gerais, Brazil) as a parameter to confirm stress of the animals, and to compare the stress procedures in respect to their hyperglycemic responses. The AMPK activity of hypothalamic tissue was evaluated at both baseline and after 20min of each stress. We have chosen to evaluate AMPK activity after 20min of stress based on our and others' previous experience showing a peak of hyperglycemic- and stress hormone-induced response around 20min[14-17]. Briefly, tissue samples were homogenized and the protein content was determined. Following immunoprecipitation with α1- and α2-AMPK antibodies AMPK activity was determined by phosphorylation of SAMS, a synthetic peptide substrate of AMPK[18]. AMPK activity was calculated using the difference of the counts between SAMS containing and SAMS negative samples and expressed as nanomoles of ATP incorporated per minute per milligram of sample of peptide.

Statistical Analysis

The data were evaluated using unpaired Student t-test or parametric (ANOVA followed by Bonferroni test) or non-parametric tests (Kruskal-Wallis test followed by Conover-Inman comparison), as appropriate. GraphPad Prism4 (Sao Francisco, CA, USA) was utilized for statistical analysis. All data are expressed as means ± standard error of the means (SEM), and significance was taken at $P < 0.05$.

Results

Fluid intake and body weight

There was no significant difference in fluid intake during the 2-week candesartan treatment between candesartan-treated group and controls (n=20 in each, 21.6±1.0 vs. 19.5±1.3 ml/100g/day respectively, P>0.05). There was no difference between the 2-week fluid intake of animals who on the day of the stress tests were assigned to stress or to control groups, suggesting that they had equal exposure to candesartan.

The initial (111.3±4.0 vs. 111.3±3.5g, P>0.05) or final (178.5±6.9 vs. 193.1± 7.6g, P>0.05) weight of candesartan-treated rats did not differ significantly from controls after two weeks treatment. However, when the weight gain was compared between groups, the candesartan-treated group showed a significantly lower weight gain than controls (67.2±5.0 versus 81.9±4.6g, P< 0.05).

Plasma Glucose Levels

There was no significant difference in plasma glucose levels in candesartan-treated non-stressed rats when compared to non-stressed controls (87.5±4.2 vs. 88.9±1.7mg/dl respectively, P>0.05, Figure 1). After 20mins restraint stress, plasma glucose levels increased significantly in both candesartan-treated group (125.8±5.1 vs. 87.5±4.2mg/dl, P<0.05) and controls (126.0±3.2 vs. 88.9±1.7 mg/dl, P<0.01). However, there were no significant differences among groups related to candesartan treatment.

In the surgical stress group, plasma glucose levels increased significantly in both candesartan-treated (83.8 ±5.3mg/dl, P<0.01), and controls (165.0±14.7 vs. 86.9±4.4 mg/dl, P<0.01, figure 1) when compared to non-stressed animals. There was no difference in the plasma glucose levels of the candesartan- treated and non-stressed control groups (83.8±5.3 vs. 86.9±4.4mg/dl, P>0.05), or the candesartan-treated and stressed control groups (163.8 ±11.1 vs. 165.0±14.7, p>0.05).

The hyperglycemic responses were different when comparing the two different stresses (Figure 1). Surgical stress under general anesthesia evoked a higher hyperglycemic stress response than restraint stress in both candesartan-treated animals and controls (163.8±11.1 vs.125.8±5.1 and 165.0±14.7 vs. 126.0±3.2 mg/dl respectively, P<0.01 for both comparisons), confirming our previous report[15].

Hypothalamic AMPK levels

In the restraint experiments, after 20min restraint hypothalamic AMPK activity increased significantly in the hypothalamus of stressed controls when compared to non-stressed controls (180.3±18.3% vs. 100.0 ±11.4% of control respectively, P<0.01), corresponding to an increase of approximately 80% as compared to baseline values. For candesartan, there was an increase in hypothalamic AMPK activity in candesartan-treated non-stressed rats when compared to non-stressed controls (163.5±17.7% vs. 100.0±11.4% of control, P<0.01, Figure 2). However, AMPK activity did not increase in stressed candesartan-treated animals after 20 min restraint when compared to candesartan-non-stressed animals (145.6±9.5% vs. 163.5±17.7% of control respectively, P>0.05). The stressed candesartan-treated animals had higher AMPK values than non-stressed controls (145.6±9.5% vs. 100.0±11.4% of control respectively, P<0.05).

In the surgical stress group, after 20min surgical stress under general anesthesia, no significant change could be observed in hypothalamic AMPK in stressed animals when compared to the non-stressed control group (95.4±12.1% vs. 100.0±10.2% of controls, $P>0.05$). However, candesartan treatment again elevated hypothalamic AMPK in the non-anesthetized, non-stressed animals (158.0±22.3% vs. 100.0±10.2% of control, $P<0.05$, Figure 2). Surprisingly, we noted a significant decrease in AMPK activity in candesartan-treated stressed group when compared to candesartan-treated non-stressed group (81.1±13.0% vs. 158.0±22.3% $p<0.01$).

Directly comparing the two types of stressor, surgical stress under general anesthesia showed lower AMPK activity than in restraint stress in both candesartan-treated animals (81.1±13.0% vs. 145.6±9.5%, $p<0.01$) and controls (95.4±12.1% vs. 163.5±17.7%, $P<0.01$).

Discussion

AMPK is a metabolic regulator sensor with extensive downstream targets involved in protein, fat, carbohydrate metabolism and energy utilization. AMPK not only senses energy status, but also functions at the tissue and organism levels to promote context-specific responses to physiological signals of metabolic status in response to ATP depletion (an increased AMP/ATP ratio) and related stimuli. Several stress stimuli have been described as activators of AMPK, such as exercise, hypoxia and ischemic stroke[19]. Hypothalamic AMPK is located in energy-sensing neurons and circuits for body energy homeostasis, and is thus positioned to function as 'master regulator' of energy balance[5]. This study is the first study, to the best of our knowledge, to address the role of hypothalamic AMPK in restraint and surgical stresses utilizing laboratory models to predict preoperative anxiety and intra-operative stress, respectively. Angiotensin II receptor blockers (ARBs) are widely used in clinical medicine, and very little is known regarding their effects on the AMPK system. We have shown here that candesartan may significantly change the hypothalamic AMPK activity at baseline as well as after restraint or surgical stress under general anesthesia, interacting in a complex fashion.

The results of this study demonstrate that (1) hypothalamic AMPK activity is increased by restraint but not changed by surgical stress under general anesthesia, that (2) hypothalamic AMPK activity is increased by the chronic administration of candesartan in non-stressed animals, and (3) chronic treatment with candesartan prevents AMPK activity further increase in restraint, and decreases AMPK activity in surgical stress under general anesthesia to values observed in non-stressed controls.

In addition to their therapeutic role in blood pressure regulation and vascular function, evidence has been accumulated regarding the beneficial effect of ARBs on metabolic regulation[20],[21], although mechanisms are yet to be clarified. Candesartan has been recently shown to increase the expression of peroxisome proliferator-activated receptor-gamma (PPARgamma) and adiponectin[22], which are both known to increase hypothalamic AMPK activity[23]. Both PPARgamma agonism and effect on adiponectin might have contributed to the observed increase on baseline hypothalamic AMPK activity[22;24;25].

Hypothalamic AMPK has emerged as an important player in the regulation of appetite, contributing to the control of energy balance metabolism at both cell and the whole body levels[5;7;18], and the anorexigenic effect of an AT1 receptor blocker with PPARgamma agonistic properties has been recently described[26]. In this study, although not specifically designed to evaluate appetite regulation, we observed a reduced weight gain in candesartan-treated animals, although initial or final weights did not differ significantly between groups. We have also observed that candesartan-treatment increased baseline AMPK activity in the liver (data not shown), in agreement to its PPARgamma agonism peripherally, and its known beneficial effects on metabolism. Alternatively, the well described candesartan-induced endothelial nitric oxide synthase (e-NOS) with augmented biovailability of nitric oxide and its consequent protection against cerebral ischemia[27;28] might also expected to be linked to this AMPK increase here shown in candesartan-treated non-stressed animals[29]. It could be worthwhile to verify if pre-surgical administration of candesartan and its elevated hypothalamic AMPK levels would be neuroprotective in intraoperative procedures utilizing temporary arterial cerebral clipping, such as those for aneurism clipping, arteriovenous malformation correction, and carotid endarterectomy procedures.

Our previously described[15] different hyperglycemic responses to both restraint and surgical stress have been reproduced in this study, corroborating to the notion of a marked heterogeneity of neuroendocrine stress responses with its specific central-activated circuit and neurochemical signature, as described by others[4;30;31]. Likewise, hypothalamic AMPK activity was here shown to respond differentially to these stressors. After restraint, control animals increased hypothalamic AMPK levels, while no change in hypothalamic AMPK levels was shown after surgery under general anesthesia. Indeed, acute restraint typically activates hypothalamic neurons in hypothalamus paraventricular nucleus[32], and this nucleus is intimately related to hypothalamic AMPK activity[33]. On the other hand, general anesthesia may prevent excess energy consumption and, therefore, theoretically spare AMPK under surgical stress. The α2 adrenoceptor agonist xylazine utilized in these experiments acts centrally to decrease efferent sympathetic activity and circulating norepinephrine concentrations, thus preserving energy consumption[34]. Ketamine, an N-methyl-D-aspartic acid receptor antagonist, has been recently demonstrated to lower brain AMPK activity when administered to rats before ischemia[35]. Thus, the anesthetic combination of ketamine and xylazine may have prevented the increase in AMPK after surgical stress in anesthetized controls, and this blockade may have been enhanced by the more marked hyperglycemic response shown on response to this stressor[33].

The role of angiotensin II AT1 receptors in the regulation of stress responses have been previously investigated[36-38]. Interestingly, it has been shown that acute restraint increases AT1 mRNA in paraventricular nucleus[39] while chronic peripheral administration of candesartan blocks brain AT1 receptors[40-42], inhibiting central responses to angiotensin II in rats[28;42;43]. Moreover, candesartan has been recently described as an anti-stress compound[44], improving stress-related disorders[45] and reducing cortisol responses to CRH stimulation[45]. Thus, the blockade of AT1 receptors in paraventricular nucleus is probably related to the absence of a further increase in hypothalamic AMPK activity in candesartan-treated animals submitted to restraint. Furthermore, the overall anti-stress effects of candesartan altogether with the aforementioned effects of anesthesia decreased hypothalamic AMPK activity in candesartan-treated animals submitted to surgical stress.

Interestingly, although hypothalamic AMPK activity is increased in candesartan-treated non-stressed controls, the treatment with this AT1 receptor blocker does not interfere with the effect of anesthesia on hypothalamic AMPK activity. Thus, in the setting of an intraoperative cerebral insult, when anesthesia is protective and therefore spares AMPK, preoperative treatment with candesartan does not seem to block this effect.

While our observations of AMPK activity in the hypothalamus following candesartan and following two types of stresses are novel, this study was not designed to specifically elucidate the underlying mechanisms. Our data demonstrate a picture of the *in vivo* stress variability of responses to common stressors, and the expanding role of AMPK in stress and anesthesia. Following policies to use the minimum necessary number of animals in experimental settings, we did not include a group of rats with anesthesia alone (without surgery), as this scenario is not relevant to clinical situations. Future studies should possibly address the molecular pathways involved in AMPK changes following these common stress events.

In summary, this *in vivo* study shows that the ARB candesartan differentially impacts hypothalamic AMPK activity, depending on whether it is at baseline or after an acute stressful event. AMPK is activated at baseline by candesartan but, AMPK is relatively or absolutely decreased after restraint and surgical stresses, and this could be due to candesartan's anti-stress properties. Furthermore, hypothalamic AMPK seems to uniquely respond to different stressors, and the combination of xylazine and ketamine was shown to prevent the AMPK stress rise and decrease AMPK activity when combined to candesartan in surgical stress. Further studies should address the importance of the association of candesartan in anaesthesia to prevent intraoperative neurological damage through alteration of hypothalamic AMPK, especially in the clinical scenario of surgical patients with metabolic syndrome. We therefore conclude that understanding the links of AMPK system to ARBs, and its modulation in stress procedures, may ultimately open a new avenue with novel treatments targeting AMPK system interactions to ARB in the reality of clinical anesthetic practice.

Figure Legends

Figure 1: Effects of restraint and surgical stresses upon plasma glucose levels in rats

treated with candesartan for 2 weeks. **p< 0.01 compared to control; •p<0.05, •• p<0.01

compared to candesartan; ♦♦ p<0.01 for comparison to control + restraint stress; ▪▪

p<0.01 for comparison to candesartan + restraint stress. (n=7-9 for each group)

Figure 2: Hypothalamic AMPK activity at baseline and after restraint and surgical stress

in rats treated with candesartan for 2 weeks. *p<0.05,**p<0.01 compared to

control; •p<0.05, ••p<0.01compared to candesartan; ♦♦ p<0.01 for comparison

to control + restraint stress; ▪▪ p<0.01 for comparison to candesartan + restraint stress

(n=7-9 for each group).

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