LEONARDO FANTINATO MENEGON

EFEITO DA ADMINISTRAÇÃO INTRACEREBROVENTRICULAR DE INSULINA SOBRE A EXCREÇÃO URINÁRIA DE SÓDIO E VIAS DE SINALIZAÇÃO DA INSULINA EM HIPOTÁLAMO DE RATOS HIPERTENSOS

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Fisiopatologia Médica, área de concentração em Medicina Experimental.

Orientador: Prof. Dr. José Antonio Rocha Gontijo

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Banca examinadora: Profº. Drº. José Antonio Rocha Gontijo
Profº. Drº. José francisco Figueiredo
Profº. Drº. Eduardo Homsi
Profº. Drº. Emmanuel de Almeida Burdmann
Profª. Drª Mirian Aparecida Boim

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Banca examinadora da tese de Doutorado

Aluno (a): Leonardo Fantinato Menegon

Orientador(a): Prof(a). Dr(a). José Antonio Rocha Gontijo

Membros:

Professor Doutor José Antonio Rocha Gontijo

Professor (a) Doutor (a) Eduardo Homsi

Professor (a) Doutor (a) José Francisco Figueiredo

Professor (a) Doutor (a) Emanuel de Almeida Burdmann

Professor (a) Doutor (a) Mirian Aparecida Boim

Curso de pós-graduação em Fisiopatologia Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

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SUMÁRIO

LISTA DE ABREVIATURAS

Akt	proteína quinase B/Akt
Cbl	produto do proto oncogene Cbl
e-NOS (e*ndotelial nitric oxide synthase*)	óxido nítrico sintase endotelial
HAS	hipertensão arterial sistêmica
IMC	índice de massa corporal (peso/altura^2)
IRS 1/2 (i*nsulin receptor substrate 1/2*)	substratos 1 e 2 do receptor de insulina
L-NAME (*N-nitro-L-arginina-metil-ester*)	inibidor da óxido nítrico sintase
LY294002	bloqueador da via da PI-3 quinase
MAPK	*mitogen-activated protein kinase*
PI-3 quinase	fosfatidilinositol 3 quinase
PKC	proteína quinase C
SH2 (*Src Homology 2*)	domínios protéicos com homologia ao proto oncogene Src2
Shc (*Src homologys and colagen homology*)	substrato do receptor de insulina
SHR (*spontaneous hypertensive rats*)	ratos espontaneamente hipertensos
UO126	bloqueador da via da MAPK
WKy (*Wystar-Kyoto*)	ratos normotensos da linhagem de Kyoto, controles genéticos dos ratos SHR

RESUMO

Evidências recentes demonstram uma estreita correlação entre situações de resistência ao efeito hipoglicemiante da insulina e conseqüente hiperinsulinemia, e hipertensão arterial sistêmica. Especula-se que a insulina possa desempenhar um papel no desenvolvimento ou manutenção da hipertensão arterial.

Embora autores demonstrem que a administração periférica de insulina diminui a excreção urinária de sódio, sugerindo uma atrativa ligação entre seu efeito renal e o desenvolvimento ou manutenção da hipertensão arterial, estudos de nosso laboratório indicam que a administração intracerebroventricular de insulina promove significativo aumento da excreção de sódio. É possível que a resistência central ao efeito natriurético da insulina promoveria um desequilíbrio na homeostase hidro-salina, favorecendo a retenção crônica de sódio.

O presente estudo foi designado para avaliar o efeito da administração intracerebroventricular aguda de insulina sobre a excreção urinária de sódio em ratos WKy e SHR, assim como caracterizar a ação direta da insulina sobre as vias de sinalização intracelulares da insulina (PI 3-quinase e MAPK) no hipotálamo destes ratos. Ainda, avaliar se a administração intracerebroventricular crônica de insulina poderia alterar a resposta dos receptores de insulina, atenuando o efeito natriurético da insulina.

Demonstramos que a administração crônica intracerebroventricular de insulina atenuou significativamente a resposta natriurética da insulina, porém não alterou a pressão arterial de ratos normotensos.

A natriurese mediada pela insulina em sistema nervoso central é regulada pela via da MAPK-ERK-1/2. Ratos SHR não apresentam incrementos na fosforilação desta via e na excreção de sódio estimulada pela insulina, caracterizando estes animais como centralmente resistentes à ação natriurética da insulina.

Um possível desequilíbrio entre a ação periférica e central da insulina sobre a regulação da natriurese nos ratos hipertensos poderia levar a alterações na homeostase hidro-salina, favorecendo a retenção crônica de sódio, contribuindo para o desenvolvimento e manutenção da hipertensão arterial.

ABSTRACT

Chronic elevated plasma insulin levels and resistance to the hypoglycemic effect of insulin have been associated with increased blood pressure in human and animal models of hypertension. This observation has led to the speculation that insulin may play a role in the development of increased blood pressure.

Although authors have shown that the peripheral action of insulin reduces urinary sodium excretion, suggesting an attractive reciprocal link between insulin's renal effect, urinary sodium excretion and the development or maintenance of hypertension, studies from our laboratory have indicated that acute intracerebroventricular injection of insulin significantly increases the output of sodium. Thus, we hypothesized that central resistance to the natriuretic effect of intracerebroventricular administration of insulin could lead to an inability of renal tubules to handle the hydroelectrolyte balance.

To test this hypothesis, we investigated the effects of acute intracerebroventricular insulin administration on sodium tubular handling in SHR and WKy, and characterized the direct actions of insulin on the PI 3-kinase and MAP-kinase in the hypothalamus of these rats. Furthermore, we investigated the long-term effect of intracerebroventricular administration of insulin on sodium tubular handling and arterial pressure of normotensive rats.

We demonstrated that chronic intracerebroventricular insulin injection attenuated urinary sodium excretion. However, this altered response did not simultaneously change the arterial blood pressure at this time.

We functionally characterized the MAPK-ERK-1/2 as the intracellular hypothalamic pathway responsible for insulin-mediated natriuresis in rats. On the other hand, our results show that central insulin administration is less effective in inducing activation of the MAPK pathway and natriuresis in SHR rats, characterizing these rats as centrally resistant to insulin-mediated natriuresis.

Thus, central resistance to the natriuretic effect of insulin could lead to an inability of renal tubules to handle the hydroelectrolyte balance, possibly contributing to the development or maintenance of hypertension.

1- INTRODUÇÃO

A hipertensão arterial sistêmica é uma doença altamente prevalente na população, afetando aproximadamente um bilhão de pessoas mundialmente, sendo responsável por cerca de sete milhões de mortes por ano (2002).

É definida clinicamente pela medida da pressão arterial sistólica acima de 140 mmHg e diastólica acima de 90 mmHg (2003). Recentemente uma reclassificação dos níveis de pressão arterial foi proposta, tendo como base resultados de estudos populacionais longitudinais, que demonstraram considerável aumento da mortalidade cardiovascular em indivíduos com pressão entre 130-139/85-89 mmHg, quando comparados a indivíduos com pressão arterial abaixo de 120/80 mmHg (VASAN, LARSON et al. 2001).

É responsável por aproximadamente 62% das doenças cerebrovasculares e 49% das cardiopatias isquêmicas, constituindo-se num importante problema médico e de saúde pública. Nas últimas décadas houve considerável aumento na porcentagem de pessoas hipertensas conscientes de sua doença, em vigência de tratamento e com níveis pressóricos controlados, assim como redução da mortalidade por acidente vascular cerebral e doença coronariana isquêmica (2003).

Sua prevalência aumenta com a idade, chegando a 50% em indivíduos com idade entre 60 a 69 anos, e aproximadamente 75% em indivíduos com mais de 70 anos (BURT, WHELTON et al. 1995).

Vários fatores de risco para o desenvolvimento da hipertensão arterial têm sido identificados: obesidade, excesso de ingestão de sódio na dieta, sedentarismo e ingestão inadequada de frutas, vegetais e potássio. A prevalência destas características nas populações é alta (STAMLER, STAMLER et al. 1999; WHELTON, HE et al. 2002).

A obesidade, definida clinicamente como ganho de peso corporal, especialmente de tecido adiposo, constitui-se num importante problema de saúde pública, atingindo diversas faixas etárias, inclusive crianças e adolescentes (KOPELMAN 2000). Sua prevalência tem crescido mundialmente nas últimas décadas, apesar dos esforços de campanhas de conscientização sobre os malefícios causados pelo excesso de gordura corporal (FLEGAL, CARROLL et al. 2002).

Estima-se que a prevalência atual de obesidade, definida por um IMC igual ou maior que 30 kg/m^2, seja de aproximadamente 30% na população adulta americana (OGDEN, CARROLL et al. 2006). Na população brasileira a prevalência de indivíduos com sobrepeso, definido por um IMC entre 25 e 29.9 kg/m^2, e obesidade, chega a aproximadamente 40%, constituindo-se atualmente na forma mais comum de má-nutrição (MONTEIRO e CONDE 1999).

Nos indivíduos obesos observa-se maior prevalência de hipertensão arterial (2001), e a perda de peso tem sido demonstrada como efetiva terapia não-farmacológica para a redução dos níveis pressóricos (1997).

As evidências que relacionam ingestão de sódio pela dieta e hipertensão arterial são crescentes. Estudos demonstram que a ingestão diária de sódio varia de acordo com a população estudada, podendo chegar a valores superiores a oito gramas por dia. A média de ingestão diária de sódio atualmente é de aproximadamente 4.1 gramas na população masculina e 2.75 gramas na população feminina americanas, enquanto em nossos ancestrais, adaptados geneticamente a um ambiente com baixa oferta de sódio, era de aproximadamente 0.1 gramas por dia (CLEVELAND, GOLDMAN et al. 1996; JAMES, RALPH et al. 1987).

A ingestão de sódio parece exercer importante influência nos níveis pressóricos em certos modelos animais, como os ratos S-Dahl, e em alguns grupos de pacientes, como os idosos, obesos, negros e diabéticos (O'SHAUGHNESSY, KARET 2004).

Adicionalmente, estudos demonstram que a redução da ingestão de sódio é capaz de reduzir a pressão arterial tanto de indivíduos hipertensos como de normotensos, independente da raça, sexo ou tipo de dieta (SACKS, SVETKEY et al. 2001; HE e MACGREGOR 2004).

O sedentarismo também tem sido implicado como fator de risco para o desenvolvimento de hipertensão arterial. Estudos demonstram que a prática regular de atividade física aeróbica é capaz de reduzir os níveis pressóricos de indivíduos hipertensos, sendo um importante item da terapia não-farmacológica da hipertensão arterial (KELLEY, KELLEY 2000; WHELTON, CHIN et al. 2002).

Apesar deste efeito salutar e da sua formal recomendação, estudos revelam que menos de 20% da população pratica atividade física regularmente (WHELTON, HE et al. 2002; STAMLER, STAMLER et al. 1999).

O papel da dieta no controle dos níveis pressóricos tem sido estabelecido recentemente. A aderência ao consumo de uma dieta rica em frutas e vegetais, com limitação do conteúdo de gordura total e de gordura saturada, também é capaz de promover redução dos níveis pressóricos de indivíduos hipertensos (SACKS, SVETKEY et al. 2001). Entretanto, o consumo diário de frutas e vegetais é considerado adequado em apenas 25% da população (STAMLER, STAMLER et al. 1999; WHELTON, HE et al. 2002).

Apesar dos recentes avanços na compreensão dos mecanismos fisiopatológicos da hipertensão, de medidas farmacológicas e não-farmacológicas para seu controle, ainda observamos um inadequado controle dos níveis pressóricos na população hipertensa, com índices crescentes de algumas complicações, como doença renal crônica e insuficiência cardíaca (2003).

1.1 – Resistência à insulina e hipertensão

Vários estudos epidemiológicos, realizados em diferentes grupos populacionais, mostram que o diabetes mellitus tipo 2 se desenvolve inicialmente pelo aparecimento de resistência à ação da insulina. Compensatoriamente há aumento da secreção de insulina pelas células pancreáticas, levando a hiperinsulinemia, para manter a glicemia em níveis normais. A hiperglicemia ocorre após a perda da capacidade de secreção de insulina pelas células β das ilhotas pancreáticas (DEFRONZO 1988).

A obesidade também se caracteriza por apresentar resistência à insulina e hiperinsulinemia compensatória (SWINBURN, NYOMBA et al. 1991; MONTAGUE, FAROOQI et al. 1997; CLEMENT, VAISSE et al. 1998).

Um importante estudo epidemiológico demonstrou uma estreita associação entre hipertensão arterial e situações clínicas de resistência ao efeito hipoglicemiante da insulina, como diabetes mellitus tipo 2 e obesidade.

Para uma mesma faixa etária e índice de massa corporal, a incidência de hipertensão arterial aumenta conforme o perfil glicêmico, sendo maior no grupo com diabetes mellitus tipo 2 do que no grupo com níveis glicêmicos normais. Da mesma forma, para uma mesma faixa etária e perfil glicêmico, a incidência de hipertensão arterial é maior no grupo de indivíduos obesos do que no grupo em que o índice de massa corporal é normal. Estima-se que cerca de 80% dos indivíduos hipertensos apresentam situações clínicas de resistência à insulina (MODAN, HALKIN et al. 1985).

A partir destas observações, vários estudos têm buscado estabelecer se há uma relação causal entre situações de resistência à ação da insulina e hipertensão arterial, ou se ambas seriam conseqüências de uma mesma predisposição genética.

1.2 – A hipertensão arterial é secundária à resistência à insulina?

Indivíduos hipertensos, não diabéticos, apresentam níveis plasmáticos de glicose e insulina maiores que indivíduos normotensos após teste de sobrecarga oral à glicose (FERRANNINI, BUZZIGOLI et al. 1987).

Quando submetidos à *clamp* euglicêmico-hiperinsulinêmico, a quantidade de glicose infundida para a manutenção da glicemia é menor no grupo de hipertensos do que no de normotensos (SHEN, SHIH et al. 1988).

Ainda, ratos SHR apresentam menor taxa de decaimento da glicose após infusão endovenosa de insulina (*Kitt*) do que seus controles genéticos normotensos, por menor fosforilação de proteínas intracelulares envolvidas na sinalização da insulina em tecido muscular (ZECCHIN, BEZERRA et al. 2003).

Estes dados sugerem que a hipertensão arterial sistêmica também pode ser considerada uma situação clínica de resistência periférica à ação da insulina.

Para avaliar se a resistência à ação da insulina é anterior à hipertensão arterial, foram estudados jovens não obesos, normotensos, filhos de pais hipertensos, com *clamp* euglicêmico-hiperinsulinêmico. Estes indivíduos, quando comparados a jovens não obesos, normotensos, cujos pais são normotensos, necessitam de menor quantidade de glicose infundida para manutenção da glicemia, demonstrando que a resistência à ação da insulina precede o desenvolvimento da hipertensão (BEATTY, HARPER et al. 1993).

Vários estudos abordaram intervenções farmacológicas e não-farmacológicas que visassem reduzir a resistência à insulina, com o intuito de avaliar se haveria concomitante redução dos níveis pressóricos.

A administração crônica de rosiglitazona a indivíduos hipertensos foi capaz de melhorar a sensibilidade à ação da insulina, além de promover redução da pressão arterial (RAJI, SEELY et al. 2003). Ainda, medidas não-farmacológicas que reduzem a resistência à ação da insulina, como exercício físico e redução do peso corporal, também promovem redução dos níveis pressóricos de indivíduos hipertensos (SHAW, GENNAT et al. 2006; NETER, STAM, et al. 2003).

Por outro lado, o tratamento da hipertensão arterial com medicamentos anti-hipertensivos não tem sido capaz de reduzir a resistência periférica à ação da insulina, sugerindo que a resistência à insulina e hiperinsulinemia não são conseqüentes à elevação pressórica. Corroborando estas evidências, a relação entre resistência à insulina e hipertensão arterial não se verifica em modelos animais ou em humanos com hipertensão arterial secundária (KOTCHEN, ZANGH et al. 1991; MARIGLIANO, TEDDE et al. 1990; REAVEN e HO 1992).

Desta forma, várias evidências sugerem que a resistência à insulina pode preceder a hipertensão arterial e colaborar para seu desenvolvimento ou manutenção, ou ainda, que uma predisposição genética poderia contribuir para o surgimento de ambas (SECHI 1999).

Como em situações de resistência à ação da insulina há hiperinsulinemia compensatória, diversos estudos têm investigado os efeitos da insulina sobre os mecanismos regulatórios da pressão arterial, com a hipótese de que a hiperinsulinemia poderia contribuir com a elevação dos níveis pressóricos ou mesmo ser a sua causa.

1.3 - Mecanismos moleculares da ação insulínica

A insulina é um potente hormônio anabólico, essencial para a manutenção da homeostase da glicose e do crescimento e diferenciação celular. É produzida pelas células β das ilhotas pancreáticas, tendo como precursor a pré-pró-insulina, um peptídeo sinalizador que direciona a cadeia peptídica para dentro do retículo endoplasmático. Neste, a pró-insulina é produzida pela clivagem do peptídeo sinalizador e formação de pontes dissulfídicas. O pró-peptídeo chega ao aparelho de Golgi, onde a insulina madura é sintetizada pela clivagem do peptídeo C, sendo então empacotada nos grânulos β e estocada na forma de hexâmeros contendo zinco.

A insulina é secretada pelas células β das ilhotas pancreáticas em resposta ao aumento dos níveis circulantes de glicose e aminoácidos após as refeições. A insulina regula a homeostase de glicose em vários níveis, reduzindo a produção hepática de glicose, através da diminuição da gliconeogênese e da glicogenólise, e aumentando a captação periférica de glicose, principalmente em tecido muscular e adiposo. A insulina também estimula a lipogênese no fígado e nos adipócitos, reduz a lipólise, bem como aumenta a síntese e inibe a degradação protéica.

A sinalização intracelular da insulina inicia-se com sua ligação a um receptor específico de membrana, uma glicoproteína heterotetramérica com atividade quinase, composta por duas subunidades α e duas subunidades β, que atua como uma enzima alostérica na qual a subunidade α inibe a atividade tirosina quinase da subunidade β. A ligação da insulina à subunidade α permite que a subunidade β adquira atividade quinase, levando a alteração conformacional e autofosforilação, o que aumenta ainda mais a atividade quinase do receptor (PATTI e KAHN 1998).

Uma vez ativado, o receptor de insulina fosforila vários substratos protéicos intracelulares em tirosina. Atualmente, dez substratos do receptor de insulina já foram identificados. Quatro desses pertencem à família dos substratos do receptor de insulina, as proteínas IRS (WHITE 1998). Outros substratos incluem Shc, Gab-1, $p60^{dok}$, Cbl, JAK2 e APS (PESSIN e SALTIEL 2000). A fosforilação em tirosina das proteínas IRS cria sítios de reconhecimento para moléculas contendo domínios com homologia a Src 2 (SH2). Dentre estas se destaca a fosfatidilinositol 3̃quinase (PI 3-quinase). A PI 3-quinase é importante na regulação da mitogênese, diferenciação celular e transporte de glicose estimulada pela insulina (FOLLI, SAAD et al. 1992; SAAD, ARAKI et al. 1992; SAAD, FOLLI et al. 1993). Atualmente, a PI 3-quinase é a única molécula intracelular considerada essencial para o transporte de glicose (CZECH e CORVERA 1999). As proteínas alvo conhecidas dessa enzima são a Akt e as isoformas atípicas da aPKC (□e □), porém a função destas proteínas no transporte de glicose ainda não está bem estabelecida (KOHN, SUMMERS et al. 1996; BANDYOPADHYAY, STANDAERT et al. 1997; KOTANI, OGAWA et al. 1998; KITAMURA, OGAWA et al. 1998; KIM, NIKOULINA et al. 1999).

Semelhante a outros fatores de crescimento, a insulina estimula a *mitogen-activated protein kinase* (MAPK). Essa via inicia-se com a fosforilação das proteínas IRS e/ou Shc, que interagem com a proteína Grb2 (PAEZ-ESPINOSA, ROCHA et al. 1999). A Grb2 está constitutivamente associada à SOS, proteína que troca GDP por GTP da Ras, ativando-a. A ativação da Ras estimula a fosforilação em serina da cascata da MAPK, estimulando a proliferação e diferenciação celular (BOULTON, NYE et al. 1991). O bloqueio farmacológico dessa via previne a ação da insulina no crescimento celular, mas não tem efeito nas ações metabólicas do hormônio (LAZAR, WIESE et al. 1995).

1.4 – Ação renal da insulina

Desde os trabalhos de Goldblatt, Wilson e Byrom, que demonstraram que lesões renais poderiam levar ao desenvolvimento de hipertensão arterial, diversos estudos têm investigado o papel do rim no controle da pressão arterial (GOLDBLATT, LYNCH et al. 1934; WILSON e BYRON 1941).

O fato de que o transplante de rins de ratos hipertensos para ratos normotensos foi capaz de promover hipertensão arterial criou o conceito de que a "hipertensão segue o rim" (BIANCHI, FOX et al. 1974). Guyton propôs que o rim de indivíduos hipertensos poderia apresentar uma inabilidade de excreção de sódio em resposta a níveis elevados de pressão arterial, conceito conhecido como "natriurese de pressão". Estes indivíduos necessitariam de níveis pressóricos mais elevados que indivíduos normotensos para manter a mesma natriurese (GUYTON, COLEMAN et al. 1974).

Desta forma, diversos estudos procuraram explorar os efeitos renais da administração de insulina e seu papel na etiopatogenia da hipertensão arterial.

A identificação de receptores de insulina em glomérulos e túbulos de ratos colocou em evidência uma possível participação da insulina no controle de funções renais (MEEZAN e FREYCHET 1980; IM, PILLION et al. 1988).

Em indivíduos normais, durante a administração endovenosa de doses fisiológicas de insulina, mantendo-se níveis normais de glicose plasmática, observou-se um declínio dose-dependente da excreção renal de sódio, sem que houvesse alteração da filtração glomerular ou da atividade do sistema renina-angiotensina-aldosterona (DEFRONZO, COOKE et al. 1975; DEFRONZO, GOLDBERG et al. 1976).

Estudos de micropunção e *clearance* de lítio demonstram que a antinatriurese é decorrente de um efeito renal distal aos túbulos proximais (COHEN, MCCARTY et al. 1989; SKOTT, HOTHER-NIELSEN et al. 1989). Como a insulina tem um efeito direto no transporte celular iônico, especula-se que sua ação antinatriurética decorra de ação tubular direta. A reabsorção de sódio pelos segmentos distais do túbulo renal apresenta estreita correlação com a atividade da Na^+-K^+-ATPase, cuja atividade é estimulada pela insulina (MOORE 1983).

Observações mais recentes demonstram que em indivíduos obesos, portadores de diabetes mellitus tipo 2 e hipertensos, a administração endovenosa de insulina, mantendo-se níveis normais de glicemia, também promoveu redução da excreção renal de sódio, em níveis semelhantes aos controles com peso corporal normal, não diabéticos e

normotensos, respectivamente. Estes estudos sugerem que apesar da resistência de diversos tecidos à ação da insulina, o tecido renal permanece sensível à sua ação antinatriurética (ROCCHIN, KATCH et al. 1989; SKOTT, VAAG et al. 1991; MUSCELLI, NATALI et al. 1996).

A influência da quantidade de sódio na dieta sobre a ação antinatriurética da insulina também foi estudada. Ratos normotensos e hipertensos foram submetidos à *clamp* euglicêmico-hiperinsulinêmico após dieta hiper-sódica, e avaliados quanto à excreção renal de sódio. Somente os ratos normotensos apresentaram abolição da antinatriurese estimulada pela insulina (SECHI 1999).

Ainda, estudou-se o efeito da dieta hiper-sódica sobre o número de receptores de insulina e nível de m-RNA do mesmo receptor em tecido renal. Somente os ratos normotensos apresentaram a esperada redução tanto do número de receptores quanto dos níveis de m-RNA, sugerindo que os ratos hipertensos perderam o mecanismo regulatório que limita a retenção de sódio quando o volume do fluído extracelular é expandido (SECHI, GRIFFIN et al. 1994).

Especula-se que o desenvolvimento ou manutenção da hipertensão arterial em condições patológicas de resistência à insulina, como diabetes mellitus tipo 2 e obesidade, poderia ser conseqüência da antinatriurese persistente, colocando em evidência uma estreita relação entre função renal e insulina quanto ao controle da pressão arterial e do metabolismo eletrolítico.

1.5- Ação vascular da insulina

A vasculatura periférica exerce importante influência na regulação dos níveis pressóricos. A insulina, além de seus efeitos metabólicos, também regula diversas funções vasculares em situações fisiológicas.

Diversos estudos têm demonstrado que a administração endovenosa de insulina promove dilatação em vasos da musculatura esquelética, aumentando o fluxo sanguíneo e diminuindo a resistência vascular, e que estes efeitos não são mediados primariamente por ativação adrenérgica ou colinérgica (ANDERSON, HOFFMAN et al. 1991; VOLLENWEIDER, TAPPY et al. 1993; RANDIN, VOLLENWEIDER et al. 1994).

Recentemente demonstrou-se que a vasodilatação mediada pela insulina em musculatura esquelética é mediada pela liberação de óxido nítrico endotelial (STEINBERG, BRECHTEL et al. 1994; SCHERRER, RANDIN et al. 1994). A insulina liga-se ao receptor de insulina nas células vasculares, ativando a via da PI3-quinase/Akt, que por sua vez promove fosforilação da óxido-nítrico sintase endotelial (e-NOS), enzima reguladora da produção de óxido nítrico, um potente vasodilatador arterial endógeno (ZENG e QUOL 1996; DIMMELER, FLEMING et al. 1999; FULTON, GRATTON et al. 1999).

Estudos em indivíduos obesos demonstram que a vasodilatação mediada pela insulina é menor que nos controles, evidenciando que a resistência à ação metabólica da insulina também existe em nível vascular (LAAKSO, EDELMAN et al. 1990; VOLLENWEIDER, RANDIN et al. 1994).

Em indivíduos com diabetes tipo 2 os dados são conflitantes. Enquanto alguns trabalhos demonstram que a resposta vasodilatadora ao estímulo com insulina está preservada (TACK, SMITS et al. 1996), outros demonstram que há resistência a este efeito (BARON, LAAKSO et al. 1991; LAAKSO, EDELMAN et al. 1992).

Indivíduos hipertensos apresentam menor relaxamento vascular dependente do endotélio após estímulo farmacológico com acetilcolina do que indivíduos normotensos (PANZA, QUYYUMI et al. 1990). No entanto, durante *clamp* euglicêmico-hiperinsulinêmico não houve diferença da vasodilatação mediada pela insulina entre indivíduos hipertensos e normotensos (ANDERSON, BALON et al. 1992; NATALI, TADDEI et al. 1997).

Por outro lado, ratos geneticamente hipertensos apresentam menor fosforilação da via da PI3-quinase/Akt/e-NOS em aorta. Especula-se que esta redução do efeito da insulina na produção de óxido nítrico endotelial poderia colaborar com o desenvolvimento ou manutenção da hipertensão arterial (ZECCHIN, BEZERRA et al. 2003).

Estes dados em conjunto apontam para um efeito pró-hipotensor da insulina, através da vasodilatação mediada pelo óxido nítrico. Em situações de resistência à ação da insulina os dados sugerem que há alteração na reposta vasodilatadora mediada pela insulina em indivíduos obesos e com diabetes tipo 2, porém nos indivíduos hipertensos a resposta está preservada (SCHERRER e SARTORI 1997).

1.6- Ação da insulina em sistema nervoso periférico

O sistema nervoso periférico exerce importante papel na regulação da pressão arterial, através da inervação de diversas estruturas envolvidas na manutenção dos níveis pressóricos: rins, adrenais, vasculatura periférica e coração. A ação da insulina no sistema nervoso periférico foi objeto de vários estudos.

Quando administrada perifericamente, durante *clamp* euglicêmico-hiperinsulinêmico, há aumento da atividade neural simpática aferida pelo aumento da concentração venosa de catecolaminas, do "*spillover*" de noradrenalina plasmática ou de registros diretos de potencial de ação por microneurografia em nervos de vasculatura de musculatura esquelética (ROWE, YOUNG et al. 1981; O'HARE, MINAKER et al. 1989; BERNE, FAGIUS et al. 1992).

No entanto, o efeito simpato-excitatório da insulina não é uniforme. Estudos demonstram que a insulina não altera a atividade simpática de rins ou adrenais em ratos normotensos (MORGAN, BALON et al. 1993).

Em alguns estudos, a administração periférica de insulina foi acompanhada de redução dos níveis pressóricos, levantando a possibilidade de que o reflexo mecanoreceptor poderia contribuir com a ativação simpática (ANDERSON, HOFFMAN et al. 1993; BARON, STEINBERG et al. 1994). É possível ainda que o mecanismo responsável pela ativação simpática seja de origem central (MUNTZEL, ANDERSON et al. 1995).

Em humanos, a administração sistêmica de insulina estimula o "*spillover*" de norepinefrina em vasculatura muscular esquelética, enquanto a infusão local de insulina em artéria do antebraço não apresenta o mesmo efeito, indicando que a ativação simpática não se faz por mecanismos locais (LEMBO, NAPOLI et al. 1992).

Em estudos com *clamp* euglicêmico-hiperinsulinêmico, existe um intervalo temporal entre o pico de concentração dos níveis plasmáticos de insulina e a atividade neural simpática, sugerindo que a insulina deve alcançar o espaço intersticial para exercer seu efeito excitatório (VOLLENWEIDER, TAPPY et al. 1993; VOLLENWEIDER, RANDIN et al. 1994).

Consistente com esta hipótese, durante a infusão de insulina em humanos, há estreita correlação entre a cinética do aumento da concentração linfática de insulina (um marcador da concentração intersticial de insulina) e do aumento da atividade neural simpática muscular. Ainda, a ativação simpática persiste após a interrupção da infusão da insulina e do declínio da sua concentração plasmática (CASTILLO, BOGARDUS et al. 1994; ANDERSON, HOFFMAN et al. 1991).

Indivíduos hipertensos, quando submetidos à *clamp* hiperinsulinêmico-euglicêmico, apresentam maior ativação do sistema nervoso simpático, aferido pela concentração plasmática de catecolaminas, do que os controles normotensos (LEMBO, NAPOLI et al. 1992).

Portanto, a insulina promove excitação do sistema nervoso simpático, de maneira não uniforme, provavelmente por um mecanismo central, podendo assim colaborar para a manutenção ou desenvolvimento da elevação pressórica nos indivíduos hipertensos.

1.7- Ação da insulina em sistema nervoso central

Até recentemente, o sistema nervoso central não era considerado como um tecido insulino-dependente. A presença de insulina no líquor e em tecido neuronal (MARGOLIS e ALTSZULER 1968; HAVRAKOVA, SCHMECHEL et al. 1978), a

presença de receptores de insulina distribuídos em diversas regiões do sistema nervoso central (HAVRANKOVA, ROTH et al. 1978), assim como a existência de um sistema de transporte que permite à insulina plasmática atravessar a barreira hemato-encefálica, colocou em evidência a participação da insulina no controle de diversas atividades neuronais (SCHWARTZ, BERGMAN et al. 1991; BAURA, FOSTER et al. 1993).

A insulina atua como substância mitogênica e promotora do crescimento neuronal fetal, assim como está relacionada ao crescimento, maturação e mielinização do sistema nervoso central (YANG, RAIZADA et al. 1981; PURO e AGARDH 1984; HEINDENREICH e TOLEDO 1989).

O papel fisiológico da insulina foi estudado através da criação de um rato com deleção do receptor de insulina neuronal. Houve desenvolvimento de obesidade, com aumento da gordura corporal, resistência à insulina e hiperinsulinemia, assim como defeitos na espermatogênese e maturação de folículos ovarianos, demonstrando que a insulina tem um importante papel no sistema nervoso central na regulação do metabolismo energético e reprodutivo (BRUNING, GAUTAM et al. 2000).

A injeção intracerebroventricular de insulina estimula a fosforilação de substratos intracelulares da via da sinalização da insulina em tecido hipotalâmico, reduzindo significativamente a ingestão alimentar (WOODS, LOTTER et al. 1979; SCHWARTZ, WOODS et al. 2000; CARVALHEIRA, RIBEIRO et al. 2003).

Adicionalmente, a injeção intracerebroventricular de metilatropina suprime a resposta insulínica a uma sobrecarga oral de glicose em ratos, demonstrando que a regulação da glicemia tem participação direta do sistema nervoso central (OHMIMA, YAMATANI et al. 1996).

A insulina também exerce efeitos no controle da atividade simpática neuronal central. Quando injetada diretamente em hipotálamo de ratos normotensos, há aumento da atividade neural simpática para tecido adiposo marrom, musculatura de membros inferiores e rins, porém não para adrenais. Esta ativação é mediada por substratos protéicos da via de sinalização da insulina em tecido neuronal, e pode ser bloqueada farmacologicamente com substâncias específicas (MUNTZEL, MORGAN et al. 2004; RAHMOUNI, MORGAN et al, 2004).

Por outro lado, há evidências de que a injeção de insulina em áreas periventriculares reduz significantemente a taxa de disparos eferentes de nervos simpáticos periféricos, e que este efeito é abolido pela destruição neuronal com injeção de ácido kaínico (SAKAGUSHI e BRAY 1987).

A insulina também participa da regulação do metabolismo hidro-salino através de efeitos diretos no sistema nervoso central. Estudos têm demonstrado que a estimulação colinérgica e adrenérgica da área septal, hipotálamo anterior lateral, órgão subfornical, assim como da porção anterior do terceiro ventrículo, promove aumento significativo da excreção de sódio. A natriurese poderia resultar de uma inibição do sistema neural simpático renal ou, indiretamente, pela ativação dos sistemas nervosos simpáticos e parassimpáticos (COVIAN, ANTUNES-RODRIGUES et al. 1975; GONTIJO, GARCIA et al. 1992).

A administração aguda intracerebroventricular de insulina promove aumento da excreção urinária de sódio em animais, efeito abolido pela denervação renal, tratamento com streptozotocina e L-NAME, enfatizando a ação da insulina no sistema nervoso central na homeostasia do sódio, bem como a necessidade de eferências renais íntegras, integridade dos receptores de insulina e do sistema do óxido nítrico para a promoção dos efeitos assinalados (AGARWALA e BAPAT 1977; MICHELLOTTO, CARVALHEIRA et al. 2002; MACEDO, FURLAN et al. 2003; FURLAN, MARSHALL et al. 2003).

No entanto, não está determinada qual a via de sinalização intracelular em sistema nervoso central que estaria envolvida na regulação da natriurese mediada pela insulina. Como já descrito anteriormente, indivíduos e modelos animais de hipertensão apresentam resistência a diversas funções estimuladas pela insulina (MONDON e REAVEN 1988).

Até o presente estudo é desconhecida a resposta do sistema nervoso central de animais hipertensos com relação à excreção de sódio mediada pela insulina. Aventamos a hipótese de que animais geneticamente hipertensos apresentam resistência central à ação natriurética da insulina, e que um desequilíbrio entre sua ação renal e central poderia colaborar para um desequilíbrio na homeostase hidro-salina, favorecendo a retenção crônica de sódio e, conseqüentemente, elevação dos níveis pressóricos.

Adicionalmente, não está determinado se a exposição crônica do sistema nervoso central a níveis elevados de insulina poderia alterar a resposta do receptor de insulina, atenuando a resposta natriurética esperada. Como o efeito antinatriurético da administração periférica de insulina está preservado em situações de resistência periférica à insulina, como diabetes mellitus e obesidade, a atenuação da natriurese centralmente mediada pela insulina poderia causar um desequilíbrio na homeostase do sódio, favorecendo a retenção de sódio e o desenvolvimento de hipertensão arterial.

2- OBJETIVOS

Os objetivos do presente estudo são:

1. Caracterizar a via de sinalização da insulina responsável pela natriurese mediada pela insulina em sistema nervoso central, em ratos normotensos, através do bloqueio farmacológico da via da PI3-quinase com LY294006 e da via da MAPK com UO126.

2. Estudar o efeito da microinjeção intracerebroventricular de insulina sobre a excreção renal de sódio em ratos espontaneamente hipertensos (SHR), seus controles genéticos normotensos (WKy), e em ratos SHR jovens (j-SHR), em idade prévia ao desenvolvimento da hipertensão.

2. Estudar o efeito da microinjeção intracerebroventricular de insulina sobre as vias de sinalização da insulina em hipotálamos de ratos hipertensos (SHR), seus controles genéticos normotensos (WKy), e em ratos SHR jovens, em idade prévia ao desenvolvimento de hipertensão.

4. Estudar os efeitos da microinjeção intracerebroventricular crônica de insulina sobre a excreção renal de sódio e sobre a pressão arterial sistêmica em ratos normotensos.

3- MATERIAIS E MÉTODOS

O planejamento experimental de cada estudo, a característica dos animais estudados, a descrição detalhada do procedimento cirúrgico para implante de cânula intracerebroventricular, os reagentes e soluções utilizados, o método utilizado para quantificação de proteínas fosforiladas por immunobloting, e outras metodologias clássicas utilizadas estão descritas detalhadamente nos trabalhos apresentados na seção de resultados.

4- RESULTADOS

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Hypertension

EFFECT OF ACUTE INTRACEREBROVENTRICULAR INSULIN MICROINJECTION ON RENAL SODIUM HANDLING IN SPONTANEOUS HYPERTENSIVE RATS (SHR)

Leonardo F. Menegon, Jose R. Tonelli, Mirian Ueno, Jose B.C. Carvalheira, Jose J.A. Saad, and Jose A.R. Gontijo

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EFFECT OF ACUTE INTRACEREBROVENTRICULAR INSULIN MICROINJECTION ON RENAL SODIUM HANDLING IN SPONTANEOUS HYPERTENSIVE RATS (SHR)

Running Title: Urinary Sodium Excretion and Intracerebroventricular Insulin Injection in SHR

[1]Leonardo F. Menegon, [1]José R. Tonelli, [2]Mirian Ueno, [2]José Barreto C. Carvalheira, [2]Mario J. A. Saad and [1]José A. R. Gontijo

[1]Laboratórios de Metabolismo Hidro-Salino e [2]Biologia Molecular, Departamento de Clinica Médica, Núcleo de Medicina e Cirurgia Experimental, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brazil

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Please address correspondence to:
J.A.R. Gontijo, Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-592 Campinas, SP, Brazil.
Phone: + 55 19 3521-8924; Fax: + 55 19 3521-8925
E-mail: gontijo@fcm.unicamp.br

ABSTRACT

Although authors have shown that the peripheral action of insulin reduces urinary sodium excretion, suggesting an attractive reciprocal link between insulin's renal effect, urinary sodium excretion and the development or maintenance of hypertension, studies from our laboratory have indicated that acute intracerebroventricular injection of insulin significantly increases the output of sodium. Thus, we hypothesized that central resistance to the natriuretic effect of intracerebroventricular administration of insulin could lead to an inability of renal tubules to handle the hydroelectrolyte balance. To test this hypothesis, we investigated the effects of acute i.c.v. insulin administration on sodium tubular handling in SHR and WKy, and characterized the direct actions of insulin on the PI 3-kinase and MAP-kinase in the hypothalamus of these rats. In the present study, we functionally characterized the intracellular hypothalamic pathway responsible for insulin-mediated natriuresis in rats. We also, show that treatment with insulin markedly increases natriuresis in WKy rats, but we do not detect insulin-induced natriuresis after pretreatment with MAPK inhibitors. On the other hand, our results show that central insulin administration is less effective in inducing natriuresis in SHR rats. Our data demonstrate an impairment of insulin activation of the MAPK pathway in these animals. Insulin-stimulated tyrosine phosphorylation of Akt, however, is similar in both phenotypes. Defective and pathway-selective insulin action in the brain may, thus, contribute to the pathogenesis of hypertension in SHR rats.

INTRODUCTION

Chronic elevated plasma insulin levels and resistance to the hypoglycemic effect of insulin have been associated with increased blood pressure in human and animal models of hypertension (1). This observation has led to the speculation that insulin may play a role in the development of increased blood pressure.

The role of the central nervous system (CNS) in the control of blood pressure and hydrosaline homeostasis has been remarkably demonstrated by several studies (2, 3). Moreover, growing evidence suggests that insulin may also modulate many brain functions, such as food intake regulation, reproductive and cardiovascular function (4-6). Observations of a selective transport of insulin across the blood-brain barrier and its selective localization in specific brain regions further support a CNS regulatory function (7, 8).

The control of several cardiovascular functions and hydrosaline homeostasis in the central nervous system is attributed to the hypothalamic areas (3, 6, 9). Furthermore, studies have recently demonstrated insulin-induced insulin receptor and post-receptor protein phosphorylation

involvement in the action of insulin in the hypothalamus of rats (10, 11). After insulin binds to the insulin receptor, intracellular protein substrates are phosphorylated, including insulin receptor substrates (IRSs – IRS-1 and IRS-2 being the most important) (12-14) and Shc (15). Following tyrosine phosphorylation, the IRSs act as docking proteins for several Src homology 2 domain-containing proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), Grb2, SHP2, Nck and Fyn (16-20). Downstream to PI 3-kinase, activation of a serine/threonine kinase, Akt occurs (21). In contrast, downstream to Grb2, activation of the mitogen-activated protein kinase (MAPK-ERK) occurs, important in the regulation of gene-expression and cell growth (22-24).

Although authors have shown that the peripheral action of insulin reduces urinary sodium excretion (25), suggesting an attractive reciprocal link between insulin's renal effect, urinary sodium excretion and the development or maintenance of hypertension, studies from our laboratory have indicated that acute intracerebroventricular (i.c.v.) injection of insulin significantly increases the output of sodium (26).

Thus, we hypothesized that central resistance to the natriuretic effect of i.c.v. administration of insulin could lead to an inability of renal tubules to handle the hydroelectrolyte balance, possibly contributing to the development or maintenance of hypertension. To test this hypothesis, we investigated the effects of acute i.c.v. insulin administration on sodium tubular handling in unanesthetized, unrestrained SHR and their age-matched genetic normotensive controls, Wystar-Kyoto rats (WKy), and characterized the direct actions of insulin on the PI 3-kinase and MAP-kinase in the hypothalamus of these rats.

MATERIALS AND METHODS

Animals. The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the study. Male, ten-to twelve-week-old SHR and WKy rats were kept in individual cages under controlled temperature (25°C) and lighting conditions (0700h-1900h), with free access to tap water and standard laboratory rodent chow (Nuvital, Curitiba, PR, Brazil): Na^+ content: 135 ± 3 μEq/g; K^+ content: 293 ± 5 μEq/g). Tail arterial pressure was estimated one day before the renal test in conscious restrained rats by the tail-cuff method, using an electrosphygmomanometer (Narco Bio-System, Austin, TX, USA). This indirect approach permits repeated measurements with a close correlation (correlation coefficient = 0.975) compared to direct intra-arterial recording. To exclude the possibility that long-term hypertension could alter the central response to insulin administration, we also investigated the effects of i.c.v. insulin injection on young, pre-

hypertensive, four-to six-weeks-old SHR rats (y-SHR). All insulin signaling experiments and renal studies were started at 8:00h.

Reagents. Human Regular Insulin (Eli Lilly and Co., Indianapolis, Indiana, USA) was stored at 4°C and diluted in saline immediately before each experiment. The PI 3-kinase inhibitor, LY294002 (Calbiochem, San Diego, CA), and the MAPK inhibitor, UO126 (LC Laboratories, MA, USA), were dissolved in dimethyl sulfoxide and further diluted in saline (0.1% dimethyl sulfoxide).

Surgical procedures. Briefly, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg^{-1} body weight) and, after loss of corneal and pedal reflexes, were positioned on a Stoelting stereotaxic apparatus. A 23-gauge guide stainless steel cannula with indwelling 30-gauge obturator was stereotaxically implanted into the lateral cerebral ventricle (LCV), using previously reported techniques and pre-established coordinates: anteroposterior, 0·2 mm from bregma, lateral 1·5 mm and vertical 4·2 mm (27). Rats were allowed one week of recovery before testing for cannula patency and position. Cannulas were considered patent and correctly positioned if a dypsogenic response was elicited after AngII injection (10).

Experimental design. After an overnight fast, each animal received a load of tap water by gavage (5% of the body weight), followed by a second load of the same volume 1 h later. Thirty minutes after the second load (basal period), insulin was microinjected in appropriate doses (3mU, 30mU and 300mU), in a volume of 3 µl, with a 10 µl Hamilton microsyringe and spontaneously voided urine was collected over a period of 120 min (experimental period) into a graduated centrifuge tube. To characterize the specific intracellular pathway that modulates natriuresis in normotensive rats, WKy rats were i.c.v. injected (3µl) with either saline (NaCl 0.15M) or specific inhibitors of the PI 3-kinase (LY294002, 50µM) or MAPK (UO126, 7µg) thirty minutes before the i.c.v. microinjection of 300mU of insulin. At the end of the experiments, blood was drawn by cardiac puncture and urine and plasma samples taken for analysis. Plasma and urine sodium concentrations were measured by flame photometry (Micronal, B262, São Paulo, Brazil), while creatinine concentrations were determined spectrophotometrically (Instruments Laboratory, Genesys V, USA). Renal clearance was calculated by a standard formula (C=UV/P), where U and P are the urinary and plasma concentrations of substances, respectively, and V is the urinary volume. Creatinine clearance was used to estimate glomerular filtration rate (GFR) and the value adjusted for body weight. Fractional sodium excretion (FE_{Na}) was calculated as C_{Na}/C_{Cr}, where C_{Na} is sodium clearance and CCr is creatinine clearance.

Blood pressure measurement. Mean arterial blood pressure was undertaken by tail plethismography in unanesthetized and awake rats. Rats were warmed in a specific cage, over a 10 min period, in a temperature of approximately 38°-39° C, to produce tail arterial vasodilatation. The animals were transferred to a restriction box, adjusted to body size, with free access to ventilation and were kept at a temperature of 38°-39° C with a ventrally positioned warmed plaque. A cuff connected to a mercury sphygmomanometer was adjusted in the proximal part of the tail, while a carbon microphone connected to a headphone was positioned at the distal part of the tail to amplify pulse sounds. The mean arterial blood pressure (MAP) was the mean of three consecutive determinations.

Western blot. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg^{-1} body weight), and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Rats were submitted to i.c.v. injection of 300mU of insulin (3µl bolus injection). After the appropriate time interval (15 minutes), the cranium was opened and the basal diencephalon, including the preoptic area and the hypothalamus, was quickly removed, minced coarsely and homogenized immediately in the solubilization buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 35 mg PMSF/ml, 10 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, and 4 mM EDTA, using a Polytron PTA 20S generator operated at maximum speed for 30s and clarified by centrifugation. Invariably, two hypothalami were pooled and 200 µg protein were used as whole tissue extract for Akt and Erk analysis or equal amounts of protein for immunoprecipitation followed by Western blot for IR and IRS-1/2 with the indicated antibodies and [^{125}I] Protein A. Quantitative analysis of the blots was performed using Scion Image software. [^{125}I] Protein A bound to the anti-phosphotyrosine and antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY) with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. The antibodies against IR (sc-711), IRS-1 (sc-559), IRS-2 (sc-8299), ERK (sc-93), p-ERK (sc-7383), Akt1 (sc-1618) and anti-phosphotyrosine (sc-508) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis. Results are expressed as means ± S.E.M. Fractional variation of renal parameters and of protein phosphorylation between basal and experimental periods were calculated and expressed as Δ%. Statistical analysis of the data was performed using one way analysis of variance for repeated measures. When the results were significant, Bonferroni´s contrast test was used to determine the extent of the differences. Paired t-test was used to compare results before and after treatments in the same group. A p value ≤ 0.05 was considered to indicate significance.

RESULTS

Figures 1-4 and Table 1 show the effects of i.c.v. dose-response insulin microinjection on renal Na^+ and K^+ handling, body weight, food intake and tail blood pressure levels in SHR, y-SHR and WKy rats, expressed as means ± SEM per 100-g b.w. All rats survived and were clinically healthy up to day five after cannula positionig in the LCV.

Effects of i.c.v injection of 3, 30 and 300 mU of insulin on renal tests of WKy, SHR and y-SHR rats - Mean arterial pressure was higher in SHR rats when compared to y-SHR and WKy (WKy: 121 ± 7 mmHg; SHR: 182 ± 12 mmHg; y-SHR: 127 ± 6 mmHg; n=15 in all groups; p=0.0001). The number of rats in each experiment was: 3mU (WKy=8; SHR=7; y-SHR=9); 30mU (WKy=7; SHR=8; y-SHR=7) and 300mU (WKy=9; SHR=10; y-SHR=10). The GFR and sodium filtered loads were similar in all groups before and after 3, 30 and 300 mU i.c.v. insulin microinjection (data not shown). As shown in Figure 1A (left), the FE_{Na} was higher in y-SHR compared to SHR and WKy groups in the basal period (WKy: 0.07 ± 0.02 %; SHR: 0.23 ± 0.04 %; y-SHR: 0.78 ± 0.16 %; p=0.000), and in the experimental period (WKy: 0.07 ± 0.01 %; SHR: 0.08 ± 0.01 %; y-SHR: 0.81 ± 0.25 %; p=0.004) after 3mU i.c.v. insulin injection. The $\Delta\%FE_{Na}$ was similar in all groups (WKy: 10 ± 25 %; SHR: -61 ± 7 %; y-SHR: 24 ± 31 %; p=0.07), as demonstrated in FIG 1.B. For the 30 mU insulin microinjection experiments, the FE_{Na} was higher in y-SHR, compared to SHR and WKy groups, both at basal (WKy: 0.07 ± 0.02 %; SHR: 0.17 ± 0.03 %; y-SHR: 0.85 ± 0.18 %; p=0.000) and during the experimental period (WKy: 0.12 ± 0.03 %; SHR: 0.10 ± 0.02 %; y-SHR: 0.89 ± 0.28 %; p=0.003), as shown in Figure 1A (middle). The $\Delta\%FE_{Na}$ was higher in the WKy group compared to SHR, but similar to that of the y-SHR group (WKy: 77 ± 38 %; SHR: -30 ± 16 %; y-SHR: 19 ± 27 %; p=0.04), as shown in FIG 1.B. In 300 mU experiments, the FE_{Na} was higher in y-SHR rats compared to others in the basal period (WKy: 0.12 ± 0.02 %; SHR: 0.26 ± 0.04 %; y-SHR: 0.94 ± 0.27 %; p=0.003), and higher in the experimental period when compared to SHR, but not WKy rats (WKy: 0.55 ± 0.11 %; SHR: 0.44 ± 0.11 %; y-SHR: 1.16 ± 0.25 %; p=0.006) as shown in Figure 1A (right). The $\Delta\%FE_{Na}$ after 300 mU insulin i.c.v. injection was higher in the WKy group compared to the SHR and y-SHR groups (WKy: 357 ± 74 %; SHR: 80 ± 37 %; y-SHR: 78 ± 45 %; p=0.001), as demonstrated in Figure 1B.

In vivo effect of i.c.v. insulin on tyrosine phosphorylation of IR in the hypothalamus - The effect of *in vivo* i.c.v. injection of 300mU of insulin on IR tyrosine phosphorylation was examined in the hypothalamus of WKy, SHR and y-SHR rats. Hypothalami from insulin or vehicle-treated rats were submitted to immunoprecipitation with anti-IR antibody and then blotted

with anti-phosphotyrosine antibody. Basal IR tyrosine phosphorylation did not differ between groups, as shown in Figure 2A. As shown in Figure 2B, insulin-stimulated IR phosphorylation was similar in all groups (WKy: 250± 30 Δ%; SHR: 195 ± 20 Δ%; y-SHR: 175 ± 20 Δ%; p=0.10). The protein expression of IR in the hypothalamus of WKy, SHR and y-SHR rats was quantitated by immunoprecipitation and immunoblotting with α-IR antibodies. The IR protein levels were not different between rats (Figure 2A, bottom).

In vivo effect of i.c.v. insulin on tyrosine phosphorylation of IRS-1/2 in the hypothalamus - Hypothalami from insulin or vehicle-treated rats were submitted to immunoprecipitation with α-IRS-1 or α-IRS-2 antibodies and then blotted with anti-phosphotyrosine antibody (Figure 2B and 2C, respectively, graphics). The basal levels of IRS-1 tyrosine phosphorylation were higher in SHR and y-SHR rats compared to WKy (WKy: 100 ± 13 %; SHR: 220 ± 15 %; y-SHR: 200 ± 10 %; p= 0.0001). As shown in FIG 2.E, the insulin-stimulated increase in tyrosine phosphorylation of IRS-1 was 200 ± 30 Δ% in WKy, 172 ± 15 Δ% in SHR and 100 ± 10 Δ% in y-SHR (p=0.009, WKy vs. y-SHR). Basal tyrosine phosphorylation of IRS-2 was similar in all groups (WKy: 100 ± 13 %; SHR: 135 ± 15 %; y-SHR: 105 ± 10 %; p=0.15). Insulin-stimulated tyrosine phosphorylation of IRS-2 was higher in y-SHR than WKy and SHR rats (WKy: 150 ± 15 Δ%; SHR: 92 ± 10 Δ%; y-SHR: 280 ± 25 Δ%; p=0.0001). The protein expression of IRS-1 and IRS-2 in the hypothalamus of WKy, SHR and y-SHR rats was quantitated by immunoprecipitation and immunoblotting with α-IRS-1 or α-IRS-2 antibodies. Protein levels of IRS-1 and IRS-2 were not different between rats (Figure 2B and Figure 2C, respectively, bottom).

In vivo effect of i.c.v. insulin on serine phosphorylation of Akt and tyrosine phosphorylation of MAP kinase (ERK-1/2) in the hypothalamus - The basal level of serine phosphorylation of Akt was higher in SHR and y-SHR compared to WKy rats, and higher in y-SHR compared to SHR rats (WKy: 100 ± 13 %; SHR: 180 ± 15 %; y-SHR: 300 ± 15 %; p= 0.000). As shown in Figure 3D, insulin i.c.v. injection was able to stimulate serine phosphorylation of Akt equally in the hypothalamus of all groups (WKy: 80 ± 10 Δ%; SHR: 66 ± 7 Δ%; y-SHR: 50 ± 8; p = 0.07). The hypothalamic protein levels of Akt were not significantly different between groups (Figure 3A, bottom). The basal levels of hypothalamic tyrosine phosphorylation of ERK-1/2 were higher in y-SHR compared to WKy and SHR rats, and higher in SHR compared to WKy rats (WKy: 100 ± 8 %; SHR; 380 ± 40 %; y-SHR: 650 ± 50 %; p= 0.0001). As shown in Figure 3E, insulin-stimulated tyrosine phosphorylation of ERK-1/2 was higher in WKy than in SHR and y-SHR rats (WKy: 200 ± 25 Δ%, SHR: 32 ± 8 Δ%; y-SHR: 31± 10 Δ%; p= 0.0001). Hypothalamic protein levels of ERK-1/2 were decreased in y-SHR rats

compared to WKy and SHR rats (WKy: 100 ± 7 %; SHR: 80 ± 5 %; y-SHR: 50 ± 4 %; p= 0.0001) as demonstrated in Figure 3C.

Effect of PI 3-kinase and MAPK blockade on renal tests of WKy rats - CCr and sodium filtered load were similar at the basal period of control and pre-treated rats with LY294002 and UO126. After insulin i.c.v. microinjection, CCr was higher in the control group compared to the previous UO126-treated group (Ins: 407 ± 10 µl/min/100g; UO126: 368 ± 11 µl/min/100g; p=0.02), but sodium filtered load was similar in both (Ins: 56.2 ± 1.1 µEq/min/100g; UO126: 49.1 ± 1.8 µEq/min/100g; p=0.51). The FE_{Na} were similar in all treatment groups at the basal time point. After 300mU of i.c.v. insulin microinjection, the LY294002 pre-treated group demonstrated a similar increase in FE_{Na} compared control rats (Ins: 338 ± 98 Δ%, n=6 vs. LY+Ins: 348 ± 77 Δ%, n=8, p=0.93), as shown in FIG 4A. However, pre-treatment with UO126, a specific inhibitor of MAP-kinase, abolished the natriuresis after insulin i.c.v. microinjection (Ins: 351 ± 101 Δ%, n=6; UO126+Ins: 78 ± 31 Δ%, n=7; p=0.02), as shown in Figure 4B.

DISCUSSION

In the present study, we functionally characterized the intracellular hypothalamic pathway responsible for insulin-mediated natriuresis in rats. We show that treatment with insulin markedly increases natriuresis in WKy rats, but we do not detect insulin-induced natriuresis after pretreatment with MAPK inhibitors. On the other hand, our results show that central insulin administration is less effective in inducing natriuresis in SHR rats. In addition, our data demonstrate an impairment of insulin activation of the MAPK pathway in these animals. Insulin-stimulated tyrosine phosphorylation of Akt, however, is similar in both phenotypes. Defective and pathway-selective insulin action in the brain may, thus, contribute to the pathogenesis of hypertension in SHR rats.

Insulin signaling in hypothalamic tissue has been recently characterized; the activation of the PI 3-kinase pathway may be involved in the regulation of food consumption by insulin (5), through a decrease in the neuropeptide Y mRNA level in the arcuate nucleus (28) and an increase in the level of pro-opiomelanocortin mRNA encoding for α-melanocyte-stimulating hormone (29). It has also been shown that hypothalamic PI 3-kinase and MAPK differentially mediate regional sympathetic activation to insulin, the first is responsible for sympathetic stimulation to the hindlimb and the second is responsible for stimulation in brown adipose tissue (30). Our results show that intracerebroventricular pretreatment with a MAPK inhibitor abolished the expected insulin-mediated natriuresis, in contrast we did not observe differences in insulin-

mediated natriuresis with a PI 3-kinase inhibitor, suggesting that centrally mediated insulin natriuresis occurs via the MAPK-ERK pathway.

The post-molecular mechanism of centrally mediated insulin natriuresis is not known; It may be mediated by a) chemical stimulation of periventricular structures related to the control of water and salt metabolism, since many brain specific natriuretic factors are located in these areas; b) influence of humoral factors that could mediate sodium excretion or c) inhibition of the efferent firing rate of peripheral sympathetic nerves (9,31). Independently of the precise hypothalamic precise mechanism of insulin-mediated natriuresis, it is possible that it acts finally via the renal nerves, since previous renal denervation abolishes sodium excretion (26).

Kidneys of hypertensive patients may be unable to excrete sodium in response to elevations in blood pressure, the so called "pressure natriuresis" (32). Thus, the level of basal natriuresis, observed in adult SHR (similar to that of normotensive rats) despite high levels of blood pressure, could be caused by the development of abnormal kidney function that impairs the excretory capability. The exact mechanism by which the kidneys lose their capacity for long term control of blood pressure is not known. Several mechanisms are hypothesized, including a reduced number of nephrons (33) and disruption in the balance of intrarenal vasoactive substances, leading to renal ischemia (34). Our results extend these data showing that SHR rats have impaired insulin-mediated natriuresis even before hypertension has been initiated.

The insulin-stimulated tyrosine phosphorylation of ERK-1/2 was blunted in both young and adult hypertensive rats, as was insulin-stimulated natriuresis, characterizing these rats as centrally resistant to insulin-mediated natriuresis. Consistent with this, is the finding in our laboratory that SHR rats exhibit an impaired natriuretic response to the intracerebroventricular injection of hyperosmotic saline, providing evidence for a down-regulation of target organ responsiveness in periventricular areas to natriuretic stimulus in this genetic hypertensive rat strain (35).

In young SHR rats, this blunted response might be the result of both a basal hyper-stimulated phosphorylation of ERK-1/2 and natriuresis, which could impair additional insulin-mediated phosphorylation and the resultant increase in sodium excretion. When these rats become adult, the basal phosphorylation of hypothalamic ERK-1/2 is diminished when compared to y-SHR, but still augmented when compared to WKy rats, but the natriuresis is similar to that found in normotensive rats. These data suggest the development of a functional impairment in the hypothalamic MAPK-ERK pathway or in the renal parenchyma to promote natriuresis in response to intracerebroventricular insulin injection.

In addition, we found that the hypothalamic protein level of ERK-1/2 is diminished in young but not adult SHR when compared to WKy rats. It is tempting to suggest that the increase in hypothalamic content of ERK-1/2 in adult SHR rats is possibly an effort to compensate for the loss of functional capacity of this via to maintain basal levels of sodium excretion.

The precise mechanism by which ERK-1/2 phosphorylation is augmented in SHR rats is not known. Studies provide evidence that the activity of the intrinsic brain renin-angiotensin system (RAS) is increased in young, pre-hypertensive, and adult SHR rats, and that this hyperactivity plays a critical role in mediating the development and maintenance of hypertension (36). Recent studies demonstrated that insulin and angiotensin II (AngII) may act through overlapping intracellular pathways, since Ang II stimulates MAPK in various tissues, including cardiac myocytes (37) and vascular smooth muscle cells (38). It is possible that hyperactivity of the brain RAS system in SHR modulates insulin intracellular pathways, increasing the basal level of ERK-1/2 phosphorylation, via a cross-talk mechanism.

The effects or insulin resistance on the MAPK pathway contrasts markedly with the ability of insulin to stimulate the PI 3-kinase pathway in the hypothalamus of SHR rats. Insulin resistance does not affect the PI 3-kinase pathway, since insulin administration increased Akt phosphorylation to the same extent in both Wky and SHR rats. Two possible reasons for this difference may be proposed: alternate insulin signaling pathways and differential signal amplification.

In summary, these data suggest that central nervous system insulin-mediated natriuresis is regulated by MAPK-ERK-1/2 pathway and that spontaneous hypertensive rats are centrally resistant to insulin-mediated natriuresis. The imbalance between peripheral and central effects of insulin in hypertensive rats could lead to a disruption in sodium homeostasis, favoring chronic sodium reabsorption and contributing to maintenance of hypertension.

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	3mU				30mU				300mU			
	Na^+ (mM)	K^+ (mM)	Cr (mg/dl)	BW	Na^+ (mM)	K^+ (mM)	Cr (mg/dl)	BW	Na^+ (mM)	K^+ (mM)	Cr (mg/dl)	BW
WKy	141±1.8	4.1±0.1	0.6±0.04	380±21[a,b]	145±1.1	4.4±0.2	0.6±0.04	353±15[a,b]	143±0.7	3.2±0.2	0.6±0.05	345±
SHR	141±0.9	3.8±0.2	0.6±0.03	284±12[c]	146±1.2	4.9±0.2	0.6±0.02	255 ± 8[c]	142±1.7	3.7±0.2	0.6±0.06	241 ±
-SHR	142±1.4	4.5±0.3	0.5±0.04	115±4	144±0.9	4.6±0.2	0.6±0.04	132 ± 5	144±0.7	4.4±0.2[b]	0.6±0.04	137 ±

Table 1.

Table 1. Effect of intracerebroventricular insulin microinjection of 3, 30 and 300mU on serum sodium, potassium and creatinine levels of WKy, SHR and y-SHR rats. BW: body weight. The data are reported as means ± SEM. $p \leq 0.05$ (ANOVA and Bonferroni´s contrast test): a) WKy vs. SHR; b) WKy vs. y-SHR; c) SHR vs. y-SHR.

Figure 1. (A) Effect of the intracerebroventricular insulin microinjection of 3mU (left), 30mU (middle) and 300mU (right) on fractional sodium excretion (FE_{Na}) of WKy, SHR and y-SHR rats. Basal is the period of thirty minutes before insulin injection while experimental is the two hour period after insulin injection. (B) Δ% FE_{Na} after i.c.v. insulin microinjection of 3, 30 and 300 mU on WKy, SHR and y-SHR rats. The data are reported as the means ± SEM. * $p \leq 0.05$.

Figure 2. (A) Insulin-induced IR tyrosine phosphorylation. Hypothalamic extracts from WKy, SHR and y-SHR rats treated with insulin or vehicle for 15 min were prepared as described in

Methods. Tissue extracts were immunoprecipitated with anti-IR antibody and immunoblotted (IB) with anti-phosphotyrosine antibody (α-pY) or as shown at the bottom, with anti-IR antibody (α-IR). The bar graph shows the quantitative tyrosine phosphorylation of IR. Data (mean ± SEM; $n = 6$) are expressed as relative to control, assigning a value of 100% to the WKy control mean. * $P < 0.05$. (B) and (C) Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2, respectively. Hypothalami from rats treated with insulin or vehicle for 15 min were lysed, and tissue extracts were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and blotted with anti-phosphotyrosine antibody (pY) or as shown at the bottom, with anti-IRS-1 (α-IRS-1) or anti-IRS-2 (α-IRS-2) antibodies. The bar graph shows the quantitative phosphorylation of IRS proteins. Data (mean ± SEM; $n = 6$) are expressed as relative to control, assigning a value of 100% to the WKy control mean. * $P < 0.05$, insulin vs. control. # $P < 0.05$, basal SHR and basal y-SHR vs. basal WKy. & $P < 0.05$, basal y-SHR vs. basal SHR. (D), (E) and (F) Δ% phosphorylation of IR, IRS-1 and IRS-2, respectively, alter i.c.v. microinjection of 300mU of insulin on WKy, SHR and y-SHR rats. * $P < 0.05$.

Figure 3. (A) Effect of insulin on serine phosphorylation of Akt. The hypothalamus was lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphoserine-specific AKT antibodies (IB, immunoblotting). Bottom: quantitative protein levels of AKT. Isolated hypothalami were homogenized, and equal amounts of protein were subjected to immunoprecipitation (IP) with αAKT, separated by SDS-PAGE, and immunoblotted (IB, immunoblotting) with the same antibody. (B) Effect of insulin on quantitative tyrosine phosphorylation of MAP-kinase (ERK-1/2) in hypothalamus of WKy, SHR and y-SHR rats *in vivo*. The hypothalamus was lysed and the proteins were separated by SDS-PAGE and blotted with phosphotyrosine-specific ERK antibodies (IB, immunoblotting). The bar graphs represent the quantitative serine phosphorylation of Akt (A) and tyrosine phosphorylation of MAP-kinase (B). Data (mean ± SEM; $n = 6$) are expressed as relative to control, assigning a value of 100% to the WKy control mean. * $P < 0.05$, insulin 300mU vs. control. # $P < 0.05$, basal SHR and basal y-SHR vs. basal WKy. & $P < 0.05$, basal y-SHR vs. basal SHR. (C) Quantitative protein levels of ERK-1/2. Isolated hypothalami were homogenized, and equal amounts of protein were subjected to immunoprecipitation (IP) with α-ERK, separated by SDS-PAGE, and immunoblotted (IB, immunoblotting) with the same antibody. (D) and (E) Δ% phosphorylation of Akt and ERK, respectively, alter i.c.v. microinjection of 300mU of insulin on WKy, SHR and y-SHR rats. * $P < 0.05$.

Figure 4. (A) Effect of the acute lateral cerebral ventricle microinjection of 300mU of insulin (INS) in LY294002 and UO126 pre-treated WKy rats on the fractional excretion of sodium (FE_{Na}). Basal is the period of thirty minutes before insulin injection while experimental is the two hour period after insulin injection. (B) Δ% FE_{Na} after 300mU of i.c.v. insulin microinjection in WKy rats pre-treated with LY294002 or OU126. The data are reported as the means ± SEM. * $p \leq 0.05$.

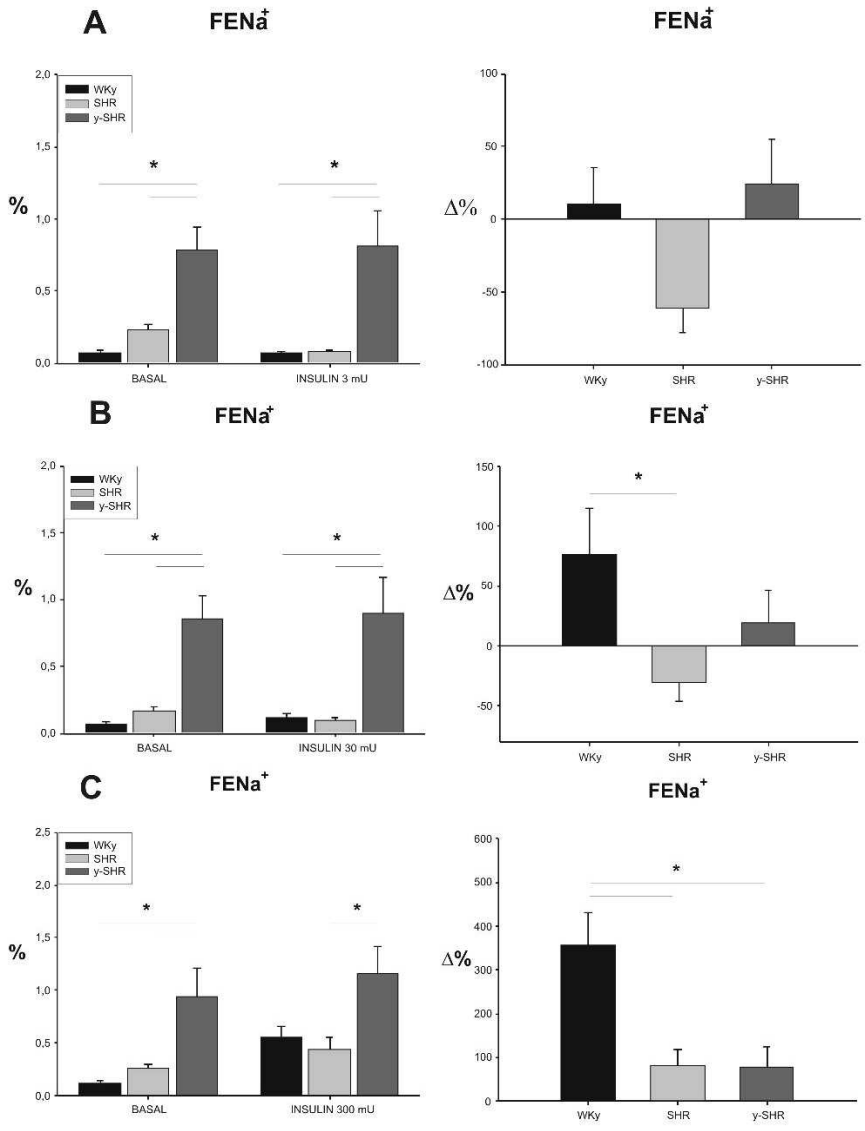
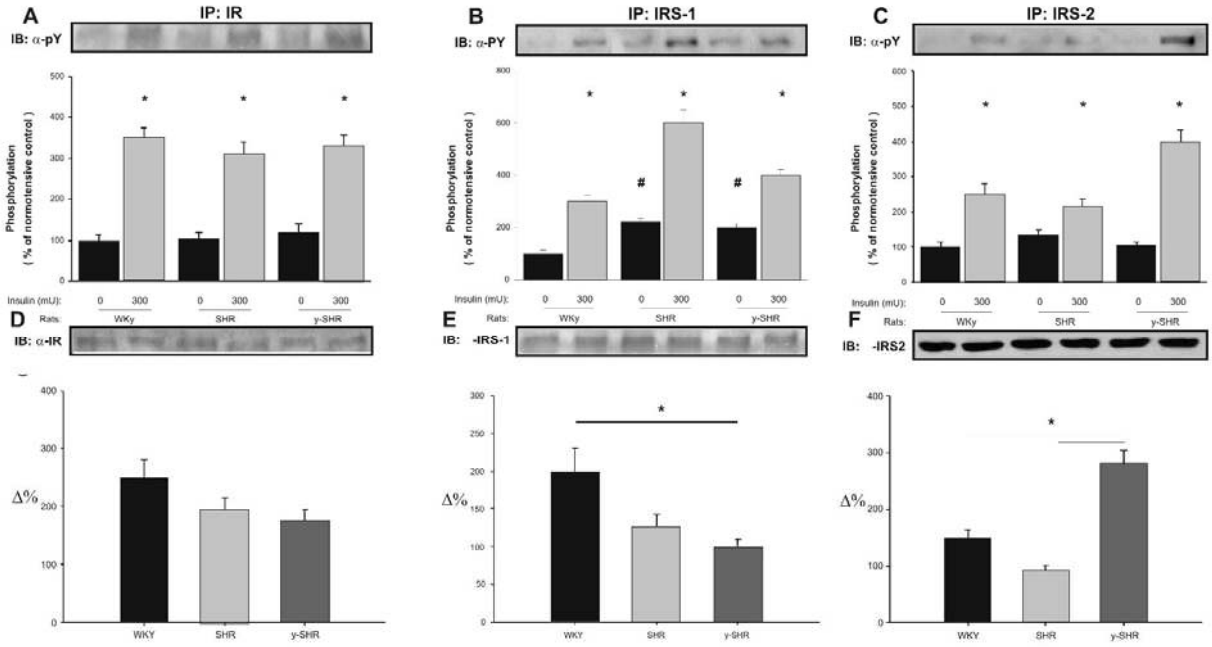


Figure 1



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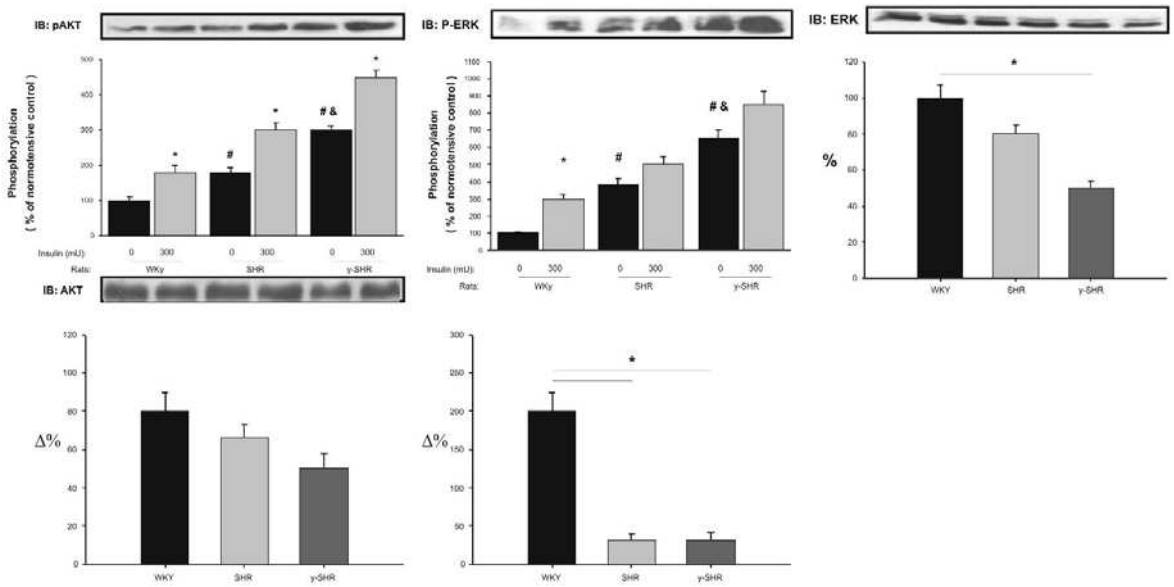

Figure 3

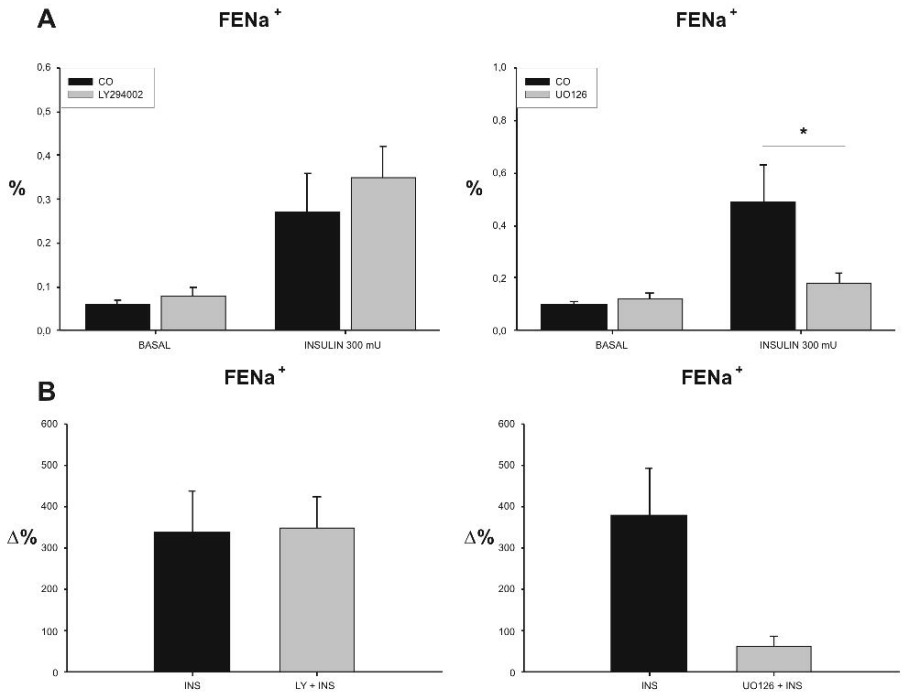
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Figure 4

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Corresponding Author: Prof. Dr. Jose AR Gontijo, MD, PhD

Corresponding Author's Institution: Unicamp

First Author: Leonardo F. Menegon, PhD

Order of Authors: Leonardo F. Menegon, PhD; Adriana Zaparolli, Master; Amanda R. Almeida, Master; Jose AR Gontijo, MD, PhD

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Abstract: ABSTRACT

The role of the central nervous system (CNS) in the control of hydrosaline homeostasis has been strikingly demonstrated by several studies. Recently, growing evidence suggests that insulin may influence the modulation of many brain functions. There are no available data regarding the CNS effect of long-term insulin injection on blood pressure and renal sodium handling, however. The long-term effect of high insulin levels in periventricular region could change the insulin receptor response, which in turn, may blunt the central natriuretic effect of insulin. This inability of renal tubules to handle the hydroelectrolyte balance may contribute to the development of arterial hypertension. In order to evaluate this hypothesis, we investigated the effects of 7-day i.c.v. insulin administration on tubular handling and blood pressure in conscious,

unrestrained rats and their controls, randomly assigned to one of two separate groups: (a) i.c.v. 0.15M NaCl-injected (n= 7) and (b) i.c.v. 42 ng.µl-1 (n= 7) insulin-injected rats. The present study shows that the percentual variation (△%) of FENa was significantly higher in 0.15M NaCl-treated rats compared to long-term insulin-treated group (Co: 440 ± 108 %, Ins: 133 ± 37 %, P=0.02). The enhanced △% of FENa in 0.15M NaCl-treated rats compared to the long-term insulin-treated group was accompanied by a significant percentual decrease in CCr variation and increased △% post-proximal sodium reabsorption rejection with unchanged FEPNa and FEK when comparing both experimental groups. The high FENa percentual variation to acute i.c.v. 42.0 ng.□ l-1 insulin injection observed in 0.15M NaCl-treated rats was blunted and significantly reduced by previous insulin i.c.v. treatment of animals for 7 days (P < 0.02). This attenuated urinary ion excretion was associated with a significant decrease in post-proximal sodium rejection. The current data suggest that a blunted renal natriuresis response to insulin periventricular region stimuli may contribute to the inability of renal tubules to handle the hydroelectrolyte balance; however, this altered response did not simultaneously change the arterial blood pressure at this time.

Cover Letter

Campinas, October 12, 2006

To Life Sciences,
B. Fredholm, M.D.
Editor-in-Chief
Tucson, Arizona
USA

Dear Editor:

Please find enclosed the original of the manuscript "Long-term effects of intracerebroventricular insulin microinjection on renal sodium handling and arterial blood pressure in rats" by Menegon et al., submitted to publish as full paper in the Life Sciences journal. Hopefully, our manuscript could be evaluated by this prestigious Journal again.

Sincerely yours,

Prof. Dr. José AR Gontijo, MD
Departamento de Clínica Médica,
Faculdade de Ciências Médicas,
Universidade Estadual de Campinas,
13083-970 Campinas, SP, Brazil
E-mail: gontijo@fcm.unicamp.br

*** Manuscript**

Long-term effects of intracerebroventricular insulin microinjection on renal sodium handling and arterial blood pressure in rats

Running Title: Urinary Sodium Excretion and Intracerebroventricular Insulin Injection

Leonardo F. Menegon, Adriana Zaparolli, Amanda R. de Almeida and José A.R. Gontijo

Disciplina de Medicina Interna, Laboratório Metabolismo Hidro-Salino, Núcleo de Medicina e Cirurgia Experimental, Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-100 Campinas, SP, Brazil.

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Correspondence:
J. A. R. Gontijo, Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-592 Campinas, SP, Brazil.
Phone: 55-19-3521 8924; FAX: 55-19-3521 8925
E-mail: gontijo@fcm.unicamp.br

ABSTRACT

The role of the central nervous system (CNS) in the control of hydrosaline homeostasis has been strikingly demonstrated by several studies. Recently, growing evidence suggests that insulin may influence the modulation of many brain functions. There are no available data regarding the CNS effect of long-term insulin injection on blood pressure and renal sodium handling, however. The long-term effect of high insulin levels in periventricular region could change the insulin receptor response, which in turn, may blunt the central natriuretic effect of insulin. This inability of renal tubules to handle the hydroelectrolyte balance may contribute to the development of arterial hypertension. In order to evaluate this hypothesis, we investigated the effects of 7-day i.c.v. insulin administration on tubular handling and blood pressure in conscious, unrestrained rats and their controls, randomly assigned to one of two separate groups: (a) i.c.v. 0.15M NaCl-injected (n= 7) and (b) i.c.v. 42 $ng.\mu l^{-1}$ (n= 7) insulin-injected rats. The present study shows that the percentual variation ($\Delta\%$) of FE_{Na} was significantly higher in 0.15M NaCl-treated rats compared to long-term insulin-treated group (Co: 440 ± 108 %, Ins: 133 ± 37 %, P=0.02). The enhanced $\Delta\%$ of FE_{Na} in 0.15M NaCl-treated rats compared to the long-term insulin-treated group was accompanied by a significant percentual decrease in C_{Cr} variation and increased $\Delta\%$ post-proximal sodium reabsorption rejection with unchanged FEP_{Na} and

FE_K when comparing both experimental groups. The high FE_{Na} percentual variation to acute i.c.v. 42.0 ng.μl^{-1} insulin injection observed in 0.15M NaCl-treated rats was blunted and significantly reduced by previous insulin i.c.v. treatment of animals for 7 days ($P < 0.02$). This attenuated urinary ion excretion was associated with a significant decrease in post-proximal sodium rejection. The current data suggest that a blunted renal natriuresis response to insulin periventricular region stimuli may contribute to the inability of renal tubules to handle the hydroelectrolyte balance; however, this altered response did not simultaneously change the arterial blood pressure at this time.

Key words: Central nervous system, intracerebroventricular, insulin, natriuresis, blood pressure and lithium clearance.

INTRODUCTION

Chronic hyperinsulinemia and peripheral insulin resistance has been associated with increased blood pressure in human and animal models of hypertension. This observation has led to the speculation that insulin may play a role in the development of increased blood pressure [Modan et al., 1985]. Conversely, the role of the central nervous system (CNS) in the control of blood pressure and hydrosaline homeostasis has been remarkably demonstrated by several studies [Brody and Johnson, 1980; Gontijo et al., 1992]. Growing evidence has suggested not only that insulin is vital to the brain but that the hormone may also exert an influence modulating many brain functions, such as food intake regulation, reproductive function, and cardiovascular function. We recently provided evidence indicating a direct and positive cross-talk between insulin and leptin at the level of Janus kinase and signal transducer and activator of transcription 3 tyrosine phosphorylation in rat hypothalamus [Carvalheira et al., 2001]. Observations of a selective transport of insulin across the blood-brain barrier and its selective localization in specific brain regions further support a CNS regulatory function. In addition, we and others have recently demonstrated insulin-induced insulin receptor and post receptor protein phosphorylation involvement in the action of insulin in hypothalamus of rats [Carvalheira et al., 2001; Fadool et., 2000]. Although it has been shown that the peripheral action of insulin reduces urinary sodium excretion, suggesting an attractive reciprocal link between insulin's renal effect, urinary sodium excretion and the development or maintenance of hypertension, studies have indicated that that acute intracerebroventricular (i.c.v.) injection of insulin significantly decreases both blood pressure and heart rate, with corresponding decreases in renal sympathetic nerve activity in anesthetized rats [Nishimura et al., 1992; Ohmima et al., 1996]. Our laboratory recently showed that centrally administered insulin produced a dose-related increase in the urinary output of sodium, which was abolished by bilateral renal denervation [Michelotto et al., 2002], nitric oxide synthase inhibition [Furlan et al., 2003] and cerebroventricular streptozotocin administration in rats [Macedo et al., 2003]. The long-term effect of high insulin levels in the periventricular region could change the insulin receptor response, which in turn, may blunt the central natriuretic effect of insulin. This inability of renal tubules to handle the hydroelectrolyte balance may contribute to the development of arterial hypertension. In order to evaluate this hypothesis we investigated the effects of long-term i.c.v. insulin administration on tubular handling and blood pressure in conscious, unrestrained rats and their controls, randomly assigned to one of two separate groups: (a) i.c.v. 0.15M NaCl-injected (n= 7) and (b) i.c.v. 42 ng.μl^{-1} (n= 7) insulin-injected rats.

MATERIAL AND METHODS

The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the study. Male Wistar-Hannover rats (220-300 g) were instrumented with an i.c.v. guide cannula and kept in individual cages under controlled temperature (25°C) and lighting conditions (0700h-1900h), with free access to tap water and standard laboratory rodent chow. Briefly, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 $mg.kg^{-1}$ body weight) and a stainless steel cannula was stereotaxically implanted into the lateral cerebral ventricle (LCV), using previously reported techniques and pre-established coordinates: antero-posterior (0.2 mm from bregma), lateral (1.5 mm from bregma) and vertical (4.0 mm from bregma). The position of the cannula was visually confirmed by 2% blue Evans infusion through the i.c.v. cannula at the end of the experiment. Seven days after the stereotaxic surgery rats were submitted to i.c.v. microinjection of 3 µl of 0.15 M NaCl (Co, n = 7) or insulin 42 $ng.\mu l^{-1}$ (Ins, n = 7), twice a day, over five days. Tap water and standard rat chow intake was recorded daily. Tail arterial blood pressure was estimated once a day before and after the 7-day treatment period, in conscious, restrained rats by the tail-cuff method, using an electrosphygmomanometer (Narco Bio-System, Austin, TX, USA). This indirect approach permits repeated measurements with a close correlation (correlation coefficient = 0.975) compared to direct intra-arterial recording [Lovenberg, 1987]. Fourteen hours before the renal test, 60 µmol LiCl $100g^{-1}$ body weight was given by gavage. After an overnight fast, each animal received a load of tap water by gavage (5% of the body weight), followed by a second load of the same volume 1-h later. Thirty minutes after the second load (basal period), insulin (100 $U.ml^{-1}$, Eli Lilly, 206 $mOsm.kg^{-1}H_20$, 42 $ng.\mu l^{-1}$) was microinjected in the volume of 3 µl, with a 10 µl Hamilton microsyringe and spontaneously voided urine was collected over a period of 120 min (experimental period) into a graduated centrifuge tube. At the end of the experiment, blood was drawn by cardiac puncture and urine and plasma samples taken for analysis. Plasma and urine sodium, potassium, and lithium concentration were measured by flame photometry (Micronal, B262, São Paulo, Brazil), while the creatinine concentration were determined spectrophotometrically (Instruments Laboratory, Genesys V, USA). Creatinine clearance was used to estimate glomerular filtration rate (GFR) and lithium clearance (C_{Li}) was used to assess proximal tubule output [Michelotto et al., 2002; Furlan et al., 2003; Macedo et al., 2003]. Fractional sodium (FE_{Na}) and potassium (FE_K) excretion were calculated as C_{Na}/C_{Cr} x 100 and C_K/C_{Cr} x 100 respectively, where C_{Na} is sodium clearance, C_K is potassium clearance, and CCr is creatinine clearance. The fractional proximal (FEP_{Na}) and postproximal ($FEPP_{Na}$) sodium excretion were calculated as C_{Li}/C_{Cr} x 100 and C_{Na}/C_{Li} x 100, respectively. Fractional variation of renal parameters between basal and experimental periods were calculated and expressed as Δ%. Statistical analysis of the data was performed using unpaired t-test for measurements between groups, and paired t-test for measurements in the same group. A p value ≤ 0.05 was considered to indicate significance.

RESULTS

All rats survived and were clinically healthy up to the 7^{th} day after a cannula was positioned in the LCV. Table 1 shows the effects of long-term i.c.v. insulin or 0.15 M NaCl microinjection on serum sodium, potassium, lithium, creatinine levels, daily rat chow and tap water intake, and arterial blood pressure before and after the i.c.v. insulin treatment,

expressed as mean ± SD per 100-g body weight. There were no significant differences between the blood pressure, daily tap water intake and serum sodium, potassium, lithium and creatinine levels (Table 1) in control i.c.v. 0.15M NaCl-injected rats, compared with the insulin-treated group. In contrast, the Table 1 demonstrates a significant decrease in the daily solid rat chow intake (Co: 14.8 ± 5.5 g *vs* Ins: 11.6 ± 6.6 g) in long-term insulin-treated rats, compared with the control i.c.v. 0.15M NaCl-injected animals.

The urinary flow rates did not differ significantly among the groups during the studies of renal tubule sodium handling (data not showed). The basal glomerular filtration rate, estimated by C_{Cr} decreases significantly in long-term insulin treated groups compared to C_{Cr} in non-treated (0.15M NaCl-treated group) rats (Fig.1). The basal FE_{Na} was significantly higher in the long-term insulin-treated group (0.053 ± 0.02 %) when compared to the 0.15M NaCl-treated group (0.007 ± 0.001 %, P=0.04), despite a significant decrease in basal C_{Cr} and FEP_{Na} parameters in insulin-treated animals (Fig 1). The enhanced basal FE_{Na} resulted in increased basal $FEPP_{Na}$ in the long-term insulin-treated group (0.27 ± 0.09 %), compared to the 0.15M NaCl-treated group (0.04 ± 0.02 %, P=0.03).

The acute i.c.v. microinjection of insulin (42.0 $ng.\mu l^{-1}$) increased the FE_{Na} in the control non-treated group from 0.007 ± 0.001 % to 0.053 ± 0.02 % (P=0.04), accompanied by a significant increase in $FEPP_{Na}$ from 0.04 ± 0.02 % to 0.27 ± 0.09 % (P=0.03) with no significant changes in C_{Cr}, FEP_{Na} and FE_K. After the long-term 3µl i.c.v. injection of 42.0 $ng.\mu l^{-1}$ insulin, the acute i.c.v. insulin administration did not significantly change the C_{Cr}, although a significant difference persists between previously treated and untreated groups (0.15M NaCl-treated: 518 ± 28 µl/min/100g *versus* Ins-treated: 351 ± 51 µl/min/100g, p=0,02) (Fig 1). Furthermore, in the previously-treated insulin animals, the acute i.c.v. microinjection of insulin (42.0 $ng.\mu l^{-1}$) increased the FE_{Na} (0.15M NaCl-treated: 0.047 ± 0.18 % *versus* Ins-treated: 0.111 ± 0.035 %), $FEPP_{Na}$ (0.15M NaCl-treated: 0.11 ± 0.03 % *versus* Ins-treated: 0.42 ± 0.15 %) and FE_K (0.15M NaCl-treated: 8.1 ± 1.2 % *versus* Ins-treated: 28.0 ± 7.9 %) (P=0.03), but did not significantly change the FEP_{Na} (see Fig. 1).

Figure 1 also shows the results of the percentual variation (Δ%) of different parameters studied. The Δ% of FE_{Na} was significantly higher in the 0.15M NaCl-treated group compared to the long-term insulin-treated group (Co: 440 ± 108 %, Ins: 133 ± 37 %, P=0,02). The enhanced Δ% of FE_{Na} in 0.15M NaCl-treated, compared to the long-term insulin-treated group was accompanied by a significant percentual decrease in C_{Cr} variation and increased Δ% post-proximal sodium reabsorption rejection with unchanged FEP_{Na} and FE_K comparing both experimental groups (Fig.1). The high FE_{Na} percentual variation to acute i.c.v. 42.0 $ng.\mu l^{-1}$ insulin injection observed in 0.15M NaCl-treated rats was blunted and significantly reduced by previous insulin i.c.v. treatment of animals during 7 days ($P < 0.02$) (see Fig 1). This attenuated urinary ion excretion was associated with a significant decrease in post-proximal sodium rejection (Fig. 1). The renal natriuretic and insulinemia response, confirming previous study [Michelotto et al., 2002], were unaffected by 42.0 $ng.\mu l^{-1}$ insulin administered SC (data not shown).

DISCUSSION

This study was designed to evaluate the effect of the long-term intracerebroventricular insulin injection on renal sodium handling and arterial pressure in rats. In the current study, we confirmed that centrally-administered insulin produced a substantial increase in the urinary output of Na^+, Li^+ and K^+, but that the response was

significantly blunted in long-term i.c.v. insulin pre-treated animals, as compared to controls. Additionally, we demonstrated that insulin-induced natriuresis occurred by increasing post-proximal tubule Na^+ rejection, despite a decreased C_{Cr} (Fig. 1) and disproportionately to the Na^+ filtered load. Thus, the observed increase in renal FE_{Na} and FE_K (Fig. 1) may be due to the inability of renal tubules to handle the electrolytes, with a promoted disruption in glomerotubular balance. To confirm this hypothesis we analyzed the normalized urinary sodium excretion comparing the basal urinary sodium excretion response after acute i.c.v. 42.0 $ng.\mu l^{-1}$ insulin administration in the long-term insulin-treated and 0.15M NaCl-treated group during experimental period and we observed that the natriuretic percentage variation ($\Delta\%$) was strikingly attenuated in the long-term insulin treated rats when compared with the control group. Thus, it is possible that the blunted urinary sodium excretion observed in insulin-treated rats after acute central insulin injection may represent a limited capacity of the rats to promote an additional enhance in the renal excretion, taking into account the already higher basal natriuretic state. The results of the present study also suggest that the natriuretic action of i.c.v insulin is preserved in chronic insulin-treated rats, however, the urinary sodium excretion after acute central insulin stimuli may be a consequence of the basal natriuresis and/or secondary to CNS insulin pathway downregulation response.

Although it has been proposed that insulin may act on the CNS to modify sympathetic outflow [Anderson et al., 1991; Lansdsberg and Krieger, 1989], a sympathetic modulator effect of centrally administered insulin on renal function has not yet been demonstrated. Authors have shown that insulin, infused into the cerebroventricular space, can reach neuronal loci after passing between ependymal cells or glial processes, to enter the interstices of the underlying cerebral neuropil [Brightham, 1968]. Injection of labeled insulin into the lateral cerebral ventricles of rats produced heavy staining in regions closer to the third ventricle [Baskin et al., 1983]. Additional findings indicated that uptake of insulin from the CSF into periventricular regions was mediated by a saturable transport system [Baskin et al., 1983]. *In vivo* and *in vitro* autoradiographic techniques have identified insulin-specific binding sites in the median eminence [Wilcox et al., 1989], the dorsomedial hypothalamus, the arcuate nucleus and the ventromedial hypothalamus [Van Houten et al., 1979].

Since a physiological role of central insulin remains to be identified, insulin binding to axonal or synaptic receptors in the CNS influences hypothalamic norepinephrine release [Sauter et al., 1983] and peripheral autonomic function [Chowers, Lavy and Halpern, 1968; Heidenreich, DeVellis, Gilmore, 1988]. Studies have shown that the injection of insulin into the periventricular area significantly reduces the efferent firing rate of peripheral sympathetic nerves and that this hypothalamic effect of insulin is abolished when neurons are destroyed by injection of kainic acid [Sakaguchi and Bray, 1987]. It has also been demonstrated that the i.c.v. injection of methylatropine suppresses the insulin responses to an oral glucose load in rats [Ohmima et al., 1996]. We, as well as others [Covian et al., 1975; Gontijo et al., 1992], have shown that cholinergic and adrenergic stimulation of the septal area, anterior lateral hypothalamus, and subfornical organ as well as the anterior portion of the third ventricle induce a dose-related natriuresis accompanied by a lesser degree of kaliuresis. All these findings led us to hypothesize that the natriuresis observed in the present study may result from either a significant and transient renal sympathetic inhibition or indirectly from a contribution of sympathetic and parasympathetic nervous system activation.

Since Claude Bernard showed, over 100 years ago, that the brain could produce diabetes in experimental animals, the CNS has been a study site of interest. The fact that hypothalamic lesions produce hyperinsulinemia and insulin resistance suggests a major role for the CNS in the regulation of insulin action and secretion. Studies from our laboratory have shown that acute intracerebroventricular insulin microinjection in rats promotes a dose-dependent increase in the sodium excretion, accompanied by a post-proximal excretion of sodium [Michelotto et al., 2002; Furlan et al., 2003; Macedo et al., 2003]. Conversely, intravenous hyperinsulinemic-euglycemic clamp and hyperinsulinemia, after the oral glucose test, in humans and rats lead to antinatriuresis [DeFronzo et al., 1975; Gontijo and Muscelli, 1996]. A previous study has also demonstrated that in conditions of insulin resistance, such as obesity, the renal antinatriuretic effect of intravenous insulin administration is preserved [Rocchini et al., 1989]. However, it is not known whether chronic central hyperinsulinemia could downregulate the central nervous system insulin receptors and, therefore, blunt the expected natriuretic response, causing a disturbance in sodium homeostasis, which in turn, would promote salt retention and hypertension development.

In fact, we also did not rule out the possibility that abnormality in the secretion of several brain humoral factors may be involved in mediating the increased natriuresis observed in the present study. It has also been shown that many brain specific natriuretic factors are located in these structures of the periventricular related to the control of water and salt balance [Covian et al., 1975; Gontijo et al., 1992; McCann et al., 1997]. Thus, chemical stimulation of these sites by insulin may induce natriuretic effects in a reproducible manner. In addition, daily rat chow intake was significantly decreased in the long-term insulin-treated group. Although there were no directs measurements of sodium balance, it is possible that chronic treatment with insulin promote a negative sodium balance, result in a lower chow intake and a higher FE_{Na}, and maintaining blood pressure at pre-treatment levels.

Although the precise mechanism responsible for the subsequent attenuated natriuretic response in centrally long-term insulin-injected rats is still unclear, the current data suggest that a blunted renal natriuresis response to insulin periventricular region stimuli may contribute to the inability of renal tubules to handle the hydroelectrolyte balance; however, this altered response did not simultaneously change the arterial blood pressure at this time. Further studies are required to investigate whether centrally insulin-induced natriuresis is altered in functional states of insulin resistance.

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Groups	Na^+ (mM)	K^+ (mM)	Li^+ (µM)	Cr (mM)	Chow intake	Water intake	BP before	BP after
Co	139 ± 1.0	3.5 ± 0.2	120 ± 3	0.6 ± 0.04	14.8 ± 5.5	27,7 ± 4,5	129 ± 2.0	123 ± 1.4
Ins	139 ± 1.6	3.7 ± 0.2	130 ± 2	0.7 ± 0.07	11.6 ± 6.6*	25,8 ± 4,6	130 ± 4.5	122 ± 1.2

Table 1. Effect of seven-day saline (Co, 0.15M NaCl-treated) or insulin (42.0 ng.µl^{-1} ins-treated) on plasmatic sodium, potassium, lithium, creatinine, daily rat chow, water intake and blood pressure (BP) before and after treatment period. The data are reported as the means ± SD per 100 g b.w.
* $P \leq 0.05$ (t-test). n = 7 in both groups.

Figure 1. Effect of the lateral cerebral ventricle microinjection of 3µl of insulin (42 ng.µl^{-1}) in five-day saline (Co) or insulin (Ins) treated rats on urinary volume, glomerular filtration rate (GFR), fractional excretion of sodium (FE_{Na}), proximal (FEP_{Na}) and post-proximal ($FEPP_{Na}$) fractional excretion of sodium and fractional excretion of potassium (FE_K) are shown in left-side. Basal is the period of thirty minutes before insulin injection while experimental is the two-hour period after insulin injection. Graphics on the right side report percentual variation (Δ%) of the experimental period compared to the basal period of each group. The data are reported as the means ± SD. * $P \leq 0.05$.

Figure

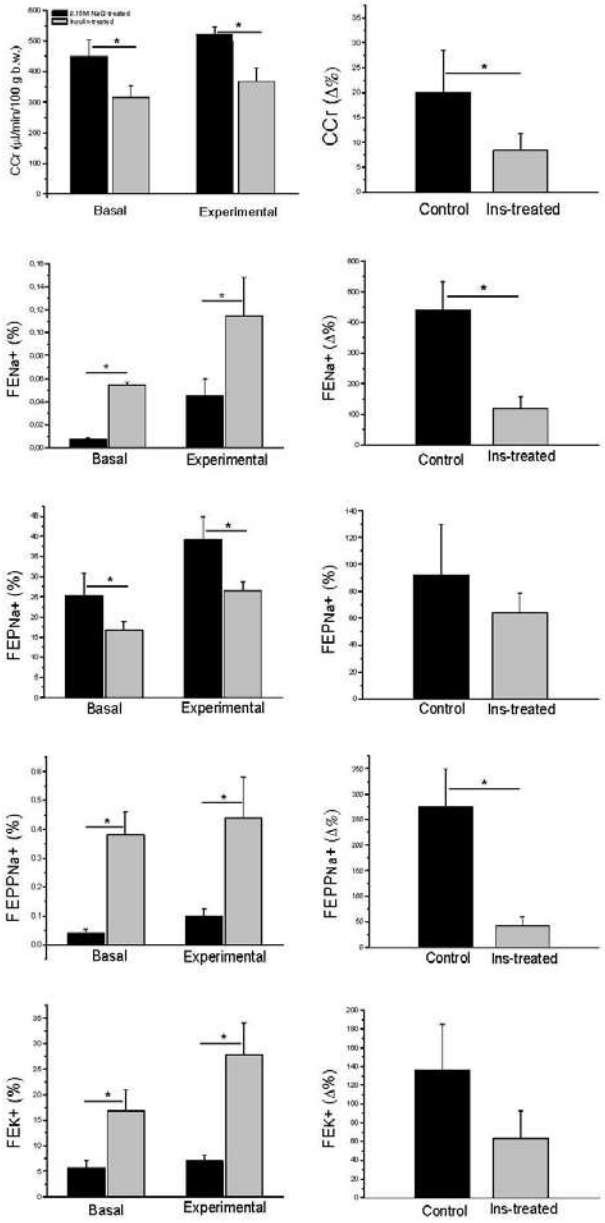

Figure 1

*** Referee List**

Petersen JS Department of Pharmacology, Panum Institute, University of Copenhagen, Denmark. jsp@zp.dk

Johnson AK Departments of Psychology and Pharmacology, University of Iowa, Iowa City, Iowa 52242-1407.

Muntzel MS Department of Biological Sciences, Lehman College, Bronx, New York 10468, USA. martinmu@alpha.lehman.cuny.edu

Kapusta DR Department of Pharmacology and Experimental Therapeutics, and the Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, 1901 Perdido Street, 70112, New Orleans, LA, USA. akapus@lsuhsc.edu

Strazzullo P Department of Clinical and Experimental Medicine, Federico II University of Naples Medical School, Via S. Pansini, 5, 80131 Naples, Italy. strazzul@unina.it

Granger JP Department of Physiology and Biophysics, University of Mississippi, Jackson, Mississippi 39216, USA. jgranger@physiology.umsmed.edu

5- DISCUSSÃO

Desde a demonstração da associação entre hipertensão arterial e situações de resistência à ação hipoglicemiante da insulina, diversos estudos têm buscado estabelecer um mecanismo fisiopatológico comum ou mesmo uma relação causal entre ambas. Como em situações de resistência à insulina há hiperinsulinemia, a insulina tem sido aventada como possível elo entre estas situações clínicas. A insulina, além de exercer papel fundamental na homeostase dos carboidratos e gorduras, também atua em diversos tecidos que participam do controle metabólico hidro-salino e da regulação da pressão arterial.

A insulina exerce suas funções através da ligação com um receptor específico de membrana e da posterior fosforilação de diversos substratos intracelulares. Dentre as principais vias de sinalização estão a da PI3-quinase/Akt e a da MAPK-ERK, responsáveis pelos efeitos metabólicos e de crescimento promovidos pela insulina.

Inicialmente o presente estudo avaliou o efeito da administração aguda intracerebroventricular de insulina sobre a excreção urinária de sódio, em ratos normotensos, após inibição específica da atividade da PI3-quinase e da MAPK, com LY294002 e UO126, respectivamente (VLAHOS, MATTER et al. 1994; FAVATA, HORIUCHI et al. 1998).

Desta maneira caracterizamos funcionalmente a via de sinalização intracelular hipotalâmica responsável pela natriurese mediada pela insulina em ratos. A injeção aguda intracerebroventricular de insulina aumentou consideravelmente a excreção de sódio nos ratos normotensos, porém esta resposta não foi detectada nos ratos tratados previamente com um inibidor da MAPK.

Recentemente, as vias de sinalização da insulina no sistema nervoso central têm sido caracterizadas. A ativação da via da PI3-quinase/Akt parece estar envolvida na regulação da ingestão alimentar, através da redução do nível de m-RNA do neuropeptídeo Y no núcleo arqueado, e do aumento do nível de m-RNA da pró-ópiomelanocortina, que regula a síntese do α-MSH (SIPOLS, BASKIN et al. 1995; BENOIT, AIR et al. 2002; CARVALHEIRA, RIBEIRO et al. 2003; XU, KAELIN et al. 2005).

Adicionalmente, tem sido demonstrado que as vias hipotalâmicas da PI3-quinase e da MAPK participam da regulação da atividade neural simpática quando estimulada pela insulina: a primeira responsável pela ativação neural em membros inferiores, e a segunda pela ativação neural em tecido adiposo marrom (RAHMOUNI, MORGAN et al. 2004).

Os mecanismos pós-moleculares responsáveis pela natriurese centralmente mediada pela insulina não estão estabelecidos. É possível que seja mediada por estimulação química de estruturas periventriculares relacionadas ao controle hídrico e salino, pois diversos fatores natriuréticos estão localizados nesta área; pela influência de fatores humorais; ou pela inibição da atividade neural simpática eferente (ANTUNES-RODRIGUES, DE CASTRO et al. 2004; SAKAGUSHI e BRAY 1987).

A despeito da definição pouco precisa quanto aos mecanismos hipotalâmicos responsáveis pela natriurese, é possível que esta seja mediada ao final pelos nervos renais, visto que a prévia denervação renal abole a excreção de sódio mediada pela injeção intracerebroventricular de insulina (MICHELOTTO, CARVALHEIRA et al. 2002).

Desta forma, os resultados de nosso estudo demonstram que a natriurese mediada centralmente pela insulina ocorre pela via da MAPK/ERK.

O presente estudo também avaliou a excreção de sódio após a injeção aguda intracerebroventricular de insulina em ratos geneticamente hipertensos. Para excluir a possibilidade de que a hipertensão crônica pudesse alterar a resposta central à administração de insulina, foram estudados ratos SHR jovens, em idade prévia ao desenvolvimento da hipertensão.

Demonstramos que os ratos SHR, tanto adultos quanto jovens, não apresentam incrementos na excreção de sódio após a administração intracerebroventricular de insulina. Da mesma forma, a fosforilação em tirosina da ERK-1/2 estimulada pela insulina está atenuada nos ratos hipertensos, caracterizando-os como centralmente resistentes à ação natriurética da insulina.

Consistente com estes dados estão achados do nosso laboratório que demonstram uma menor resposta natriurética à administração intracerebroventricular de salina hiperosmolar em ratos hipertensos, sugerindo que há uma menor responsividade de áreas periventriculares a estímulos natriuréticos nesta linhagem de ratos (GUADAGNINI e GONTIJO 2006).

Nos ratos hipertensos jovens é possível que a resposta natriurética atenuada após a administração central de insulina seja resultado tanto de uma fosforilação basal de ERK-1/2 quanto de uma natriurese basal elevadas, que poderiam resultar em uma incapacidade de incrementos adicionais na fosforilação desta via e no conseqüente aumento da excreção de sódio.

Guyton colocou em evidência o papel dos rins no controle crônico da pressão arterial e sugeriu que rins de indivíduos hipertensos apresentariam uma inabilidade na excreção de sódio em resposta a elevações da pressão arterial (GUYTON, COLEMAN et al. 1974). A natriurese observada nos ratos hipertensos adultos, em níveis semelhantes à dos normotensos, corrobora esta hipótese e evidencia o desenvolvimento de uma incapacidade funcional renal de excreção de sódio em resposta ao estímulo pressórico.

O exato mecanismo pelo qual o rim perde a capacidade excretória não é conhecido. Especula-se que possa ser decorrente de um menor número de néfrons, tendo em vista que diversas patologias que cursam com redução do número de néfrons e da área de superfície de filtração, também evoluem com hipertensão arterial (BRENNER, GARCIA et al. 1988). Estudos demonstram que ratos SHR têm um número menor de glomérulos quando comparados a ratos normotensos (SKOV, NYENGAARD et al. 1994).

Ainda, especula-se que um possível desequilíbrio entre substâncias vasoativas intra-renais, que resultaria em isquemia do parênquima renal, poderia colaborar para o desenvolvimento da perda da capacidade excretória de sódio e, conseqüentemente, da hipertensão (JOHNSON, HERRERA-ACOSTA et al. 2002).

Nos ratos SHR adultos a fosforilação basal da ERK-1/2 está diminuída em relação aos jovens, mas continua aumentada em comparação aos ratos normotensos. No entanto, a excreção basal de sódio é semelhante entre os ratos SHR adultos e normotensos. Estes dados sugerem que os ratos SHR adultos, além da inabilidade excretória renal, também apresentam uma alteração funcional na via hipotalâmica da MAPK-ERK.

Ainda, os níveis protéicos hipotalâmicos de ERK-1/2 estão diminuídos nos ratos SHR jovens, porém não nos adultos, quando comparados aos normotensos. É possível que o aumento da quantidade protéica de ERK-1/2 no hipotálamo dos ratos hipertensos adultos seja uma tentativa de compensar a perda da capacidade funcional desta via em manter níveis basais elevados de excreção de sódio.

O mecanismo preciso pelo qual a fosforilação basal da ERK-1/2 está aumentada nos ratos hipertensos não é conhecido. Estudos demonstram que a atividade do sistema renina-angiotensina cerebral está aumentada em ratos hipertensos jovens e adultos, e que esta hiperatividade teria importante participação no desenvolvimento a na manutenção da hipertensão arterial (VEERASINGHAM e RAIZADA 2003).

Estudos recentes demonstram que angiotensina II e insulina podem agir por vias de sinalização intracelulares comuns. A angiotensina II é capaz de estimular a fosforilação da via MAPK-ERK em vários tecidos, como células musculares lisas vasculares e cardiomiócitos (EGUCHI, NUMAGUCHI et al. 1998; CARVALHEIRA, CALEGARI et al. 2003).

Em certas áreas do sistema nervoso central de ratos hipertensos, como núcleo paraventricular e medula ventro-lateral rostral, há aumento da atividade da PI3-quinase modulada pelo sistema renina-angiotensina, visto que o tratamento com um inibidor da enzima conversora da angiotensina foi capaz de reduzir a atividade da PI3-quinase (VEERASINGHAM, YAMAZATO et al. 2005).

Postula-se que a atividade da PI3-quinase em certas áreas do sistema nervoso central seja modulada pelo sistema renina-angiotensina e esteja envolvida na regulação da pressão arterial através de um mecanismo que envolve o metabolismo de catecolaminas cerebrais (YANG e RHAIZADA 1999; LU, YU et al. 1996).

É possível que a hiperatividade do sistema renina-angiotensina cerebral dos ratos hipertensos module a sinalização intracelular da insulina, aumentando a fosforilação basal da ERK-1/2 em hipotálamo, através de um mecanismo de "cross-talk".

Recentemente, estudo realizado em nosso laboratório, ainda não publicado, demonstrou que a administração intracerebroventricular de angiotensina II promove uma menor resposta natriurética em ratos SHR adultos, quando comparados a animais WKy de mesma idade (ZAPPAROLI et al., 2007). No entanto a resistência observada na via da MAPK contrasta com a habilidade da insulina em estimular a via da PI3-quinase, visto que a fosforilação da Akt está preservada no hipotálamo destes ratos.

Em resumo, os dados do presente estudo sugerem que a natriurese mediada pela insulina em sistema nervoso central é regulada pela via da MAPK-ERK-1/2 e que ratos hipertensos são centralmente resistentes à natriurese mediada pela insulina. O desequilíbrio entre a ação periférica e central da insulina sobre a regulação da natriurese nos ratos hipertensos poderia levar a alterações na homeostase hidro-salina, favorecendo a retenção crônica de sódio e contribuindo para a manutenção ou desenvolvimento da hipertensão arterial.

Outra questão a ser elucidada é se o efeito crônico da hiperinsulinemia em regiões periventriculares poderia alterar a resposta dos receptores de insulina e, conseqüentemente, reduzir o efeito natriurético central mediado pela insulina.

Demonstramos que a resposta natriurética à administração central de insulina está reduzida em ratos previamente tratados com insulina intracerebroventricular.

Tanto em animais como em cultura de células a estimulação crônica com insulina promove dessensibilização e diminuição da regulação da sinalização insulínica (INOUE, CHEATHAM et al. 1996). Isto se deve em parte pela internalização e degradação

do receptor de insulina, assim como por diminuição da atividade de substratos intracelulares, como o IRS-1 (REED, NEWTON et al. 1989; KNUTSON 1991; RICE, LIENHARD et al. 1992; CHEATHAM, SHOELSON et al. 1993).

A exposição crônica à insulina também resulta em redução da sua principal ação metabólica, o transporte de glicose mediada pelo transportador de glicose de superfície celular (GARVEY, OLEFSKY et al. 1985).

Ainda, a atividade da MAPK não é estimulada pela insulina em cultura de células submetidas a tratamento crônico com insulina (INOUE, CHEATHAM et al. 1996). Da mesma forma, ratos diabéticos e hiperinsulinêmicos apresentam menor ativação do complexo MAPK/ERK estimulado pela insulina em musculatura esquelética, do que seus controles (HEI, CHEN et al. 1995).

Como a excreção basal de sódio está aumentada nos ratos submetidos a tratamento crônico com insulina em relação aos controles, podemos inferir que a insulina mantém sua capacidade de estimular a natriurese, mesmo após estímulo crônico. No entanto, é possível que a hiperinsulinemia crônica central cause uma dessensibilização da via de sinalização da insulina responsável pela natriurese, limitando incrementos adicionais na excreção de sódio mediada pela insulina.

Como descrito acima, a insulina também exerce efeitos no controle da atividade simpática neuronal central. Quando injetada diretamente em áreas hipotalâmicas de ratos normotensos, é capaz de aumentar a atividade neural simpática para diversos tecidos (MUNTZEL, MORGAN et al. 2004; RAHMOUNI, MORGAN et al, 2004).

O desequilíbrio entre uma menor ação central natriurética e um efeito persistente ativador do sistema nervoso simpático, incluindo uma elevada atividade eferente simpática renal, poderia, pelo menos em parte, explicar a menor resposta natriurética obtida no presente estudo.

A manutenção da sensibilidade renal ao efeito antinatriurético da insulina em situações de hiperinsulinemia crônica, associada à redução da resposta natriurética central após estímulo insulínico crônico de regiões periventriculares poderia promover um desequilíbrio na homeostase hidro-salina, favorecendo a retenção crônica de sódio, o que poderia colaborar para a manutenção ou desenvolvimento da hipertensão arterial.

Os dados referentes aos efeitos da hiperinsulinemia crônica sobre a pressão arterial são conflitantes. No presente estudo não foi observado aumento da pressão arterial nos ratos tratados cronicamente com insulina. Porém, a administração de insulina foi restrita ao sistema nervoso central. Como a injeção intracerebroventricular de insulina não altera a insulinemia plasmática, não houve ação direta da insulina em tecido renal que pudesse estimular a antinatriurese (MICHELOTTO, CARVALHEIRA et al. 1992).

Estudo realizado em cães para avaliar os efeitos da administração crônica intra-renal de insulina, independente de seu efeito sistêmico, demonstrou que apesar de uma redução transitória da excreção de sódio, não houve elevação dos níveis pressóricos (HALL, BRANDS et al. 1991).

Estudos em cães demonstram que a administração crônica endovenosa de insulina, por até vinte e oito dias, em níveis comparáveis àqueles observados em animais obesos, não foi capaz de elevar a pressão arterial em animais normais, em animais com aumento da atividade simpática secundária à infusão crônica de angiotensina II ou norepinefrina, em animais submetidos à dieta hiper-sódica ou mesmo naqueles em que a massa renal foi reduzida cirurgicamente (HALL, BRANDS et al. 1990; HALL, COLEMAN et al. 1990; KOOPMANS, OHMAN et al. 1997).

Ainda, um estudo que avaliou indivíduos com insulinoma, um tumor produtor de insulina, e que apresentavam hiperinsulinemia primária, demonstrou que não houve elevação dos níveis pressóricos (SAWICKI, HEINEMANN et al. 1992).

Por outro lado, ratos submetidos à dieta hiper-lipídica apresentaram elevação dos níveis pressóricos após oito semanas de tratamento, associado ao desenvolvimento de obesidade e hiperinsulinemia, e a um significativo aumento da atividade simpática, como demonstrado pela elevação da excreção renal de catecolaminas. Porém, a prevenção de obesidade e conseqüente hiperinsulinemia por restrição da quantidade de caloria ingerida, aboliu o efeito hipertensor da dieta hiper-lipídica (KAUFMAN, PETERSON et al. 1991).

Em outro estudo, a administração crônica endovenosa de insulina, com manutenção de níveis normais de glicemia, promoveu aumento da pressão arterial, independente da quantidade de sódio ingerido pela dieta (BRANDS, HILDEBRANDT et al. 1992).

Estes dados em conjunto sugerem que a hiperinsulinemia isolada não é capaz de elevar os níveis pressóricos e que fatores adicionais podem estar envolvidos na etiopatogênese da hipertensão.

Em síntese, os estudos realizados demonstraram que:

1. A natriurese mediada pela insulina em sistema nervoso central é regulada pela via da MAPK-ERK-1/2.

2. Os ratos da linhagem geneticamente hipertensa, tanto adultos quanto jovens, não apresentam incrementos na excreção de sódio e na fosforilação em tirosina da ERK-1/2 estimulada pela insulina, sugerindo que estes animais se caracterizam pela resistência central à ação natriurética da insulina.

3. Um possível desequilíbrio entre a ação periférica e central da insulina sobre a regulação da natriurese nos ratos hipertensos poderia levar a alterações na homeostase hidro-salina, favorecendo a retenção crônica de sódio e contribuindo para o desenvolvimento e a manutenção da hipertensão arterial sistêmica.

4. A administração crônica intracerebroventricular de insulina atenuou significativamente a resposta natriurética da insulina, porém não alterou a pressão arterial de ratos normotensos.

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