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EFEITO DA DEFICIÊNCIA E SUPLEMENTAÇÃO COM MAGNÉSIO SOBRE A TOLERÂNCIA À GLICOSE, SENSIBILIDADE À INSULINA E NAS ETAPAS INICIAIS DA SINALIZAÇÃO DA INSULINA EM RATOS

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CAPÍTULO 3 – Magnesium deficiency modulates the insulin signaling pathway in liver but not in muscle of rats.

RESUMO GERAL

Vários estudos têm demonstrado que o magnésio desempenha papel importante na homeostase glicêmica. Assim, no presente trabalho foi avaliado o efeito da deficiência de magnésio, da sua duração e da interação entre estes dois fatores na secreção e ação da insulina. Ratos foram alimentados com dieta deficiente em magnésio durante 6 (DF-6) ou 11 (DF-11) semanas e comparados com ratos alimentados com dieta controle durante os mesmos períodos de tempo (CO-6 e CO-11). Os ratos dos grupos DF-6 e DF-11 apresentaram níveis de magnésio sérico mais baixos do que os CO-6 e CO-11, porém a homeostase glicêmica entre os grupos DF-6, CO-6 e CO-11 não foi diferente. Os animais do grupo DF-11 apresentaram maior velocidade de decaimento da glicose (Kg) e redução da área total sob a curva de glicose, quando comparados aos animais dos grupos CO-6, CO-11 e DF-6, assim como menor área total sob a curva de insulina quando comparados com os ratos CO-11 e DF-6, indicando um aumento da sensibilidade à insulina. Com a finalidade de avaliar o efeito da suplementação de magnésio, ratos alimentados com dieta controle ou com dieta deficiente durante 6 semanas receberam, ao final desse período e durante 5 semanas adicionais, uma dieta suplementada com magnésio (grupos SCO e SDF, respectivamente). No grupo SDF os níveis de magnésio sérico, a velocidade de decaimento da glicose e áreas totais sob as curvas de glicose e insulina, não foram significativamente diferentes daqueles apresentados pelo grupo CO-11, indicando que a suplementação com magnésio evitou o aumento da sensibilidade à insulina. Não foi verificada diferença na velocidade de decaimento da glicose (Kitt) durante o teste venoso de tolerância à insulina, assim como na sinalização da insulina no músculo e fígado dos grupos DF-6 e CO-6. Entre os grupos alimentados durante 11 semanas, o DF-11 apresentou maior velocidade de decaimento da glicose, enquanto que este mesmo parâmetro nos grupos SDF e SCO não se diferenciou dos ratos CO-11. Nenhuma diferença foi observada na sinalização da insulina no músculo dos animais dos grupos CO-11, DF-11 e SDF. No fígado de ratos DF-11, o nível protéico e o grau de fosforilação do receptor de insulina e do substrato-1 desse receptor apresentaram-se aumentados, assim como verificou-se maior associação entre o substrato-1 do receptor e a subunidade p85 do fosfatidilinositol 3-quinase, quando comparados com ratos CO-11. Nenhuma diferença foi encontrada nas etapas iniciais da ação da insulina nos grupos SDF e CO-11. Estes resultados sugerem que as mudanças nas etapas iniciais da transmissão do sinal insulínico no fígado, induzidas por diferentes níveis séricos de magnésio, podem ter um papel fundamental na homeostase glicêmica.

SUMMARY

Numerous studies have demonstrated a major role for magnesium in insulin action and secretion. Therefore, we investigated the effect of Mg deficiency, duration of feeding, and the interaction between these factors on the secretion and action of insulin. Rats fed a Mg-deficient diet for 6 (DF-6) or 11 (DF-11) weeks, and rats fed a control diet for the same periods (CO-6 and CO-11 groups) were compared. DF-6 and DF-11 rats had serum Mg levels lower than the control groups, but no change in glucose homeostasis was observed among DF-6, CO-6 and CO-11 rats. DF-11 rats showed a greater glucose desappearance rate (Kg) and a reduced total area under the glucose curve compared to CO-6, CO-11 and DF-6 rats, as well as a reduced total area under the insulin curve compared to the CO-11 and DF-6 rats, indicating increased sensitivity to insulin. In order to evaluate the effect of supplementation, rats fed a control or Mg-deficient diet for 6 weeks were then fed a Mg-supplemented diet for 5 weeks (SCO and SDF groups, respectively). In the SDF rats, the serum Mg levels, glucose disappearance rate and total area under the glucose and insulin curves were restored to control values, indicating that the Mg supplementation prevented the increase in sensitivity to insulin. No differences were found in the glucose desappearance rate during an i.v. insulin tolerance test (Kitt), as well as in the insulin signaling in muscle and liver from DF-6 and CO-6. Among the groups of rats fed for 11 weeks, the DF-11 group had a significantly greater glucose desappearance rate while the rate of the SDF and SCO groups did not differ from CO-11 rats. No differences were observed in muscle insulin signaling of rats from the CO-11, DF-11 and SDF. In DF-11 rats, insulin receptor and insulin receptor substrate-1 protein and phosphorylation levels were elevated in liver and there was a greater association between the insulin receptor substrate-1 and p85 subunit of phosphatidylinositol 3-kinase compared with CO-11 rats. No diferences were found in the early steps of insulin action in SDF and CO-11 rats. These results suggest that the changes in the early steps of insulin signal transduction in the liver, induced for different serum Mg levels, may play an important role in the glucose homeostasis.

INTRODUÇÃO GERAL

Magnésio (Mg) é o segundo cátion intracelular mais abundante. Ele é cofator de aproximadamente 300 reações enzimáticas, principalmente aquelas que utilizam ligação fosfato de alta energia. Dessa forma, ele participa de inúmeros processos metabólicos, inclusive daqueles ligados ao metabolismo de carboidratos que estão envolvidos na regulação da secreção e ação da insulina.

Baseados em dados epidemiológicos que relacionam o descontrole glicêmico de pacientes diabéticos tipo 2 com a reduzida concentração de Mg eritrocitário, assim como em estudos *in vitro*, alguns autores atribuíram ao Mg uma função crítica no metabolismo da glicose sugerindo que a sua deficiência seria um estado associado ao desenvolvimento da resistência à insulina.

No entanto, essa relação causa-efeito é contestada por evidências sobre a resistência à insulina como agente da deficiência de Mg no diabetes tipo 2 como também, pela observação de fenômenos ligados ao diabetes que poderiam levar à deficiência do cátion. Somado a isso, vários estudos com modelos animais de deficiência de Mg relatam uma maior captação de glicose, contrariando a sugestão sobre a importante função do Mg na ação da insulina.

Por outro lado, alguns autores acreditam que diferentes períodos de deficiência de Mg possam exercer diferentes efeitos sobre o metabolismo do cátion e, conseqüentemente, sobre o metabolismo da glicose.

Assim, apesar de ainda não se dispor de dados conclusivos relacionados às alterações no metabolismo de Mg que ocasionariam a maior tolerância à glicose, vários mecanismos têm sido propostos na tentativa de explicar as alterações que ocorrem na homeostase da glicose durante a deficiência de Mg.

Entre esses mecanismos estão o aumento da sensibilidade periférica e/ou hepática à insulina, ou mesmo maior captação não-insulino dependente, sendo que para qualquer uma dessas hipóteses, não se tem conhecimento sobre os mecanismos moleculares, da sinalização da insulina ou não, que poderiam estar envolvidos nessas alterações.

OBJETIVOS

Dessa forma, considerando que a hipomagnesemia possa estar relacionada ao aumento da sensibilidade periférica à insulina; sabendo que o Mg é cofator de sistemas enzimáticos envolvidos em ligações fosfato de alta energia e, que as proteínas quinases são responsáveis por etapas de sinalização, no presente trabalho de tese tivemos como objetivo geral avaliar o efeito da deficiência e da suplementação com magnésio sobre a ação da insulina em ratos. Para tanto, foram estabelecidos os seguintes objetivos específicos:

1. Verificar *in vivo* a tolerância à glicose e a sensibilidade à insulina exógena utilizando, para tanto, ratos submetidos à dieta deficiente de Mg durante diferentes períodos (6 e 11 semanas).
2. Verificar os efeitos da suplementação com magnésio sobre a tolerância à glicose e a sensibilidade à insulina exógena in vivo, utilizando, para tanto, ratos previamente submetidos à dieta deficiente em Mg.
3. Verificar os passos iniciais da via de sinalização da insulina no fígado e músculo de ratos deficientes e suplementados com magnésio.

CAPÍTULO 1

ALTERAÇÕES NO METABOLISMO DA GLICOSE NA DEFICIÊNCIA DE MAGNÉSIO

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ALTERAÇÕES DO METABOLISMO DA GLICOSE NA DEFICIÊNCIA DE MAGNÉSIO

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Magnésio, deficiência, insulina, glicose.

Magnésio e homeostase glicêmica – Magnesium and glucose homeostasis

RESUMO

Magnésio (Mg) é um cátion essencial que age como cofator para adenosina trifosfatases em muitas reações enzimáticas. Vários estudos mostram seu envolvimento na ação e secreção de insulina, além dos efeitos desse hormônio sobre o metabolismo e transporte do Mg. Entretanto, os resultados são conflitantes. Alguns autores sugerem que a deficiência de Mg está implicada direta ou indiretamente com a resistência à insulina no diabetes mellitus, enquanto outros descrevem uma relação inversa, ou ainda, que a deficiência de Mg levaria ao aumento da captação de glicose. A interação do Mg com outros íons, os mecanismos hormonais e neuro-hormonais compensadores e, possivelmente a duração da deficiência, são alguns dos fatores descritos como os responsáveis pelas variações na regulação glicêmica observadas durante a deficiência de Mg.

ABSTRACT

Magnesium (Mg) is an essencial cation involved in many enzymatic reations as a cofactor to adenosine triphosphatases. Several studies have documented the role of Mg in the insulin action and secretion and the effects of insulin on Mg metabolism and transport. These results, however, are unclear. Mg deficiency could be one of the causes of insulin resistance in diabetes mellitus or, inversely, could be one of the consequences of it. Moreover, an increased glucose disposal is suggested. An interaction between Mg and other ions, hormonal and neurohormonal compensating mechanisms and probably the duration of the deficiency are some factors responsible for changing glucose homeostasis in the Mg deficiency.

REVISÃO BIBLIOGRÁFICA

Diversas evidências sugerem que o magnésio desempenhe um papel relevante na homeostase da glicose. Magnésio, o segundo cátion intracelular mais abundante, serve como cofator para aproximadamente 300 reações enzimáticas, principalmente aquelas que utilizam ligações fostato de alta energia. Entre elas são de especial importância as proteínas quinases, enzimas que catalizam a transferência do fosfato γ do ATP-Mg para o substrato protéico. O Mg liga-se à molécula de ATP para formar um substrato verdadeiro ou pode tornar lábil a ligação fostato terminal do ATP para facilitar sua transferência para outras moléculas. Além disso, o Mg pode servir para neutralizar as cargas negativas na molécula de ATP e facilitar sua ligação à enzima envolvida na reação. As proteínas quinases compõem uma família diversificada com mais de 100 enzimas (ELIN 1994), algumas das quais responsáveis por etapas de sinalização.

Devido a essas funções, atualmente muitos estudos vêm procurando estabelecer uma relação causa-efeito entre a deficiência Mg e a resistência à insulina. Pacientes com DM tipo II (diabetes mellitus não-insulino dependente), uma doença multifatorial na qual a resistência à insulina é reconhecidamente uma das causas do controle glicêmico deficitário, freqüentemente apresentam um estado de hipomagnesemia (LEGRAND et al.1987). A causa para tal estado não está esclarecida, havendo evidências que indicam deficiência alimentar desse cátion, enquanto outras apontam anormalidades no seu metabolismo como conseqüência da resistência insulínica (DURLACH et al.1993).

Estudos epidemiológicos descrevem que a ingestão deficiente de magnésio é um fator de risco para o desenvolvimento do DM tipo II, independentemente da idade, índice de massa corporal, ingestão de álcool e história familiar da doença (COLDITZ et al. 1993). Menor ingestão alimentar de magnésio foi verificada também em pacientes com DM tipo I (diabetes mellitus insulino dependente) (DJURHUUS et al. 1994), embora muitos autores concordem que pacientes com DM tipo II, com pobre controle metabólico, mais do que aqueles do tipo I, sejam freqüentemente afetados pelas mudanças nos níveis de magnésio plasmático e eritrocitário (SJÖGREN et al.1988).

Por outro lado, alguns autores descrevem que no diabetes mellitus a deficiência de Mg representa um caso de depleção, e não simplesmente de menor ingestão. Ela resulta de mecanismos complexos que afetam a absorção do cátion, sua distribuição entre o compartimento extracelular e as várias partes do compartimento intracelular e, o aumento da excreção urinária de Mg. Entre esses mecanismos complexos, os que mais interferem no metabolismo desse mineral são: os efeitos diretos e indiretos da deficiência de insulina, a deficiência endógena de vitamina D, talvez, a deficiência de piridoxina e a perda de taurina, como também, os efeitos iatrogênicos, tais como, altas doses de insulina e biguanidas e doses farmacológicas de vitaminas B e C. Aparentemente, a severidade do diabetes, mais do que sua existência, contribui para o desenvolvimento da deficiência de Mg no diabetes (DURLACH 1988).

No diabetes mellitus, a ação osmótica da glicose contribuiria para a hipermagnesúria, uma vez que a reabsorção tubular de magnésio está reduzida na hiperglicemia (SJÖGREN et al. 1988). Estudos em animais descreveram um defeito tubular renal específico para o Mg no diabetes, o qual, junto com a diurese osmótica, seria o responsável pela grande perda desse mineral (LEVY et al. 1994). A causa desse defeito é desconhecida, mas pode ser relacionada à prolongada hiperglicemia, insulinopenia, distúrbio no metabolismo de fosfato, ou outras mudanças hormonais características da doença (GARLAND 1992).

Evidências sobre a resistência à insulina como agente indutor da deficiência intracelular de Mg foram apresentadas por PAOLISSO et al. (1988), a partir de observações *in vitro*. A insulina aumentou a concentração de magnésio eritrocitário em indivíduos normais e pacientes com DM tipo II, porém, nos diabéticos a curva dose-resposta deste efeito foi desviada para a direita, com significativa redução do efeito máximo da insulina sobre o acúmulo de magnésio. Resultado semelhante foi verificado em índios Pima não diabéticos quando, em resposta à insulina, apresentaram menor acúmulo de magnésio eritrocitário em relação a índios Caucasianos. Os autores atribuíram tal redução ao alto grau de resistência à insulina freqüentemente observado nesses índios (PAOLISSO & RAVUSSIN, 1995). Assim, se a captação tecidual de magnésio é normalmente regulada pela insulina, então a resistência à insulina poderia causar ou exacerbar a deficiência intracelular de magnésio (ALZAID et al, 1995). O envolvimento de um processo mediado

pelo receptor de insulina no transporte de Mg foi observado por HWANG et al. (1993), ao estudar o efeito da insulina sobre as mudanças do Mg intracelular em plaquetas de humanos sadios. Através de pré-incubação com insulina e plasma rico em plaquetas hirudinizadas, os autores observaram um efeito dose e tempo dependentes sobre o aumento do magnésio intracelular, medido em células marcadas com Mag-fura 2, através de espectrofotometria de fluorescência. Na presença de anticorpo monoclonal anti-receptor de insulina, o efeito da insulina foi abolido, sugerindo que o mecanismo de transporte era um processo mediado pelo receptor de insulina. Além disso, o transporte de magnésio estimulado pela insulina foi inibido pela adição de 5 mmol de EDTA (ácido etilendiaminotetracético), enquanto que o binding do receptor não foi afetado; portanto, o magnésio foi transportado do meio extracelular.

A insulina, entre outros hormônios, tem sido reconhecida como uma importante substância regulatória do balanço de magnésio. LOSTROH & KRAHL (1973, 1974) demonstraram que a insulina adicionada *in vitro* promove aumento no acúmulo de magnésio e potássio nas células do músculo liso do útero de ratas. Os autores sugerem que a insulina, após se ligar ao seu receptor da membrana plasmática, possa afetar uma bomba ATPase aumentando a entrada de magnésio e potássio na célula. Em outro estudo *in vitro*, demonstrou-se que o acúmulo de Mg eritrocitário na presença de 5 mmol/l de glicose e 100 mU/l de insulina foi totalmente abolido pela ouabaína (um inibidor específico da Na^+ K^+ ATPase), enquanto que a adição só de glicose não teve efeito significativo. Aparentemente, uma bomba ATPase está envolvida nos mecanismos pelos quais a insulina regula o conteúdo de Mg eritrocitátio (PAOLISSO et al.1986).

A redução do transporte de Mg pode ter implicações no mecanismo de resistência à insulina e na patogênese das complicações no diabetes. Alterações iônicas intracelulares de Ca e Mg, medidas através de resonância magnética nuclear, foram observadas em eritrócitos maduros de humanos normais, quando crescentes doses de insulina resultaram no aumento de Ca e Mg livres de maneira dose e tempo dependentes. O reduzido nível de Mg associado à elevação de Ca intracelular, que são observados na hipertensão essencial e DM tipo II, portanto, decorreriam de uma dissociação do balanço iônico, onde o acúmulo de Ca estimulado pela insulina predominaria sobre a ação insulínica de promover a captação de Mg

(BARBAGALLO et al. 1993). Este maior influxo de Ca e reduzida concentração intracelular de Mg poderia, então, causar ou exacerbar a resistência à insulina nos pacientes com DM tipo II (ALZAID et al. 1995). A elevação dos níveis de cálcio livre celular, provocada pela deficiência de Mg em humanos, tem sido relatada como uma das causas da resistência à insulina (DRAZNIN et al. 1988), assim como, pequenas alterações na razão Ca/Mg nas células β pancreáticas foram implicadas na redução da secreção de insulina observada em ratos deficientes em Mg (SUÁREZ et al. 1995).

O efeito da deficiência de Mg aumentando a atividade de enzimas gliconeogênicas, fosfoenolpiruvato carboxiquinase (PEPCK) e frutose-1,6-bifosfatase (FDPase) (McNEILL et al. 1982), embora sem uma forte correlação com o aumento da secreção de hormônios contra-reguladores, tem sido relacionado com o aumento da produção hepática basal de glicose (LOWNEY et al.1995). SANUI & RUBIN (1978) ratificaram o conceito anterior (LOSTROH & KRAHL, 1974) sobre o magnésio como segundo mensageiro na ação da insulina. Através de estudos *in vitro* utilizando fibroblastos embrionários de galinha, SANUI & RUBIN (1978) descreveram que os efeitos metabólicos da insulina dependeram de mudanças no transporte e conteúdo catiônico celular e não do inverso. Para TONYAI et al. (1985) o baixo conteúdo de Mg intracelular aumenta a microviscosidade da membrana, prejudica a interação da insulina e seu receptor reduzindo a ação da insulina. Tal afirmação não foi comprovada por SUÁREZ et al. (1993) ao constatar que o binding de insulina no músculo esquelético de ratos hipomagnesêmicos manteve-se semelhante aos animais controle, enquanto que a atividade tirosina quinase do receptor de insulina nesse tecido estava reduzida. Outros autores, porém, acreditam que a resistência insulínica induzida pela deficiência em Mg seja distal à entrada da glicose na célula (KANDEEL et al. 1996).

Embora vários estudos epidemiológicos e experimentais monstrem que a deficiência em Mg está associada a mudanças na homeostase glicêmica, seus resultados são conflitantes.

Pacientes com diabetes mellitus, por exemplo, frequentemente apresentam ambas as deficiências em Mg e K. Dados sobre a relação entre a deficiência de Mg e de K mostram que a deficiência de Mg isolada pode não alterar o metabolismo de carboidratos, porém, a tolerância à glicose torna-se insatisfatória se associada à deficiência de K (DURLACH &

COLLERY 1984, WHANG et al. 1969). Na verdade, a deficiência de K e P, comuns no diabetes, são fatores que diretamente induzem à deficiência em Mg (DURLACH 1988).

Um efeito semelhante ao da insulina, observado em estudos com cães e diafragma isolado de ratos, foi atribuído à deficiência de Mg (KAHIL et al. 1966, 1968). Os cães deficientes apresentaram maior captação de glicose em relação aos seus controles e os diafragmas de ratos deficientes em Mg, incubados sem ou em baixa concentração de insulina, tiveram, respectivamente, 3 ou quase 2 vezes maior captação de glicose do que o diafragma dos ratos controles. Quando os diafragmas foram incubados em concentração máxima de insulina, a captação de glicose foi similar entre os ratos deficientes e os controles. Em contraste, a adição de Mg ao meio livre desses íons reduziu a captação de glicose nesse tecido dos animais deficientes em Mg, enquanto que a captação pelo diafragma dos animais controles não foi afetada pela presença de Mg no meio (KAHIL et al. 1968).

Consistente com o estudo em cães (KAHIL et al. 1966), o índice de desaparecimento da glicose (Kg) durante teste intravenoso de tolerância à glicose (IVGTT), foi aumentado significativamente em ratos deficientes em Mg (LEGRAND et al. 1987, REIS et al.**submetido**). Resultados similares foram observados em estudos utilizando administração intraperitoneal de glicose (GUEUX & RAYSSIGUIER 1983) ou oral de sacarose (KIMURA et al. 1996). Em adição, vários estudos relataram que ratos deficientes em Mg apresentaram níveis de insulina basal ou a área sob a curva de insulina (ΔI) significativamente menores do que aqueles não deficientes, enquanto que os níveis glicêmicos basais mantiveram-se semelhantes aos dos ratos controles ou a área sob a curva de glicose (ΔG) foi significativamente menor (LEGRAND et al. 1987, LOWNEY et al. 1988, 1990, McNEILL et al. 1982, REIS et al. **submetido**).

Embora esses resultados sugiram que a sensibilidade à insulina seja aumentada pela deficiência de Mg, alguns autores propõem a hipótese de uma maior captação não-insulino dependente. Nos estudos conduzidos por KAHIL et al. (1968), ratos deficientes em Mg e normais foram tornados diabéticos através de injeções de anticorpo anti-insulina. Ambos os grupos tiveram significativas elevações da glicemia, porém, a concentração de glicose sangüínea nos ratos diabéticos deficientes em Mg foi aproximadamente 15% menor do que nos diabéticos controles. A captação de 2-DG (2-deoxiglicose) pelo diafragma isolado de

ratos deficientes em Mg e agudamente diabéticos foi de 90% dos ratos não diabéticos deficientes em Mg. Por outro lado, a captação de glicose nos ratos diabéticos não deficientes em Mg foi de 64% dos controles não diabéticos e não deficientes em Mg.

Outros dados da literatura ratificam esta hipótese ao descrever que: 1) a captação de glicose estimulada pela insulina manteve-se inalterada, enquanto que 2) a captação basal foi aumentada em ratos deficientes em Mg (LOWNEY et al. 1995), 3) baixos níveis glicêmicos foram mantidos, apesar de reduzida insulinemia e menor quantidade e grau de translocação do transportador de glicose dependente de insulina (GLUT-4) (KIMURA et al. 1995), ou ainda, 4) o receptor de insulina (IR) e o substrato-1 do receptor de insulina (IRS-1) do músculo gastrocnêmio de ratos deficientes em Mg, quando estimulados com 10^{-5} M de insulina, não apresentaram alteração no nível protéico, grau de fosforilação ou na associação IRS-1 com a enzima fosfatidilinositol 3-quinase (PI 3-quinase) (REIS et al. 2000), embora o índice de desaparecimento da glicose durante teste de tolerância à glicose (Kg), fosse significantemente maior do que o verificadado nos ratos controles (REIS et al. **submetido**).

Os tecidos e os mecanismos que contribuem para o proposto aumento na captação de glicose não dependente de insulina na deficiência de Mg são desconhecidos. Supõem-se que a atividade e/ou o número de transportadores de glicose não dependentes de insulina (GLUT-1 e GLUT-3) estejam aumentados.

O aumento na captação de glicose mediada pela insulina em concentrações basais foi também proposto (LOWNEY et al. 1995), porém, o fígado e o músculo gastrocnêmio de ratos deficientes em Mg não apresentaram, ao nível basal de insulina, alterações na concentração e no grau de fosforilação das proteínas envolvidas nos passos iniciais da sinalização da insulina (REIS et al. 2000).

Embora não haja dados conclusivos sobre as alterações na sensibilidade periférica à insulina em animais deficientes em Mg (KAHIL et al. 1968, LOWNEY et al. 1995, STEFIKOVA et al. 1992), o aumento da sensibilidade hepática ao hormônio, proposto por LOWNEY et al. (1995), pode estar relacionado ao aumento nos níveis protéicos e graus de fosforilação do receptor de insulina (IR), do substrato-1 do receptor de insulina (IRS-1), e no aumento da associação IRS-1 com a enzima fosfatidilinositol 3-quinase (PI 3-quinase) no fígado de ratos deficientes em Mg, estimulados com insulina na concentração de 10^{-5} M

(REIS et al. 2000). No entanto, dados da literatura sugerem, indiretamente, que na deficiência de Mg a captação de glicose nos tecidos periféricos envolva mecanismos diferentes daqueles que necessitam da ativação e translocação de transportadores de glicose-4 (GLUT-4). Estudos com cultura de adipócitos em meio deficiente de Mg mostraram que não havia diferença no transporte de 2-DG, estimulado pela insulina, apesar de reduzidas oxidação da glicose a CO_2 e incorporação da glicose em triacilgliceróis (KANDEEL et al. 1996). Em adição, a menor quantidade e grau de translocação do GLUT-4 no tecido adiposo de ratos deficientes de Mg, frente a reduzidas concentrações plasmáticas de glicose e insulina, poderiam ratificar essa hipótese (KIMURA et al.1995).

O envolvimento do magnésio na síntese e secreção de insulina pelas células β têm sido discutido. Reduzidas áreas sob a curva de insulina, observadas em ratos deficientes em Mg e submetidos a testes de tolerância à glicose, sugerem menor secreção de insulina e/ou maior clearance hepático, ou ainda, menor reserva pancreática do hormônio (LEGRAND et al. 1987, LOWNEY et al. 1995). Estudos em pâncreas perfundido de rato demonstraram que variações na concentração de magnésio extracelular modularam a liberação de insulina induzida pela glicose, sendo que altos níveis de magnésio levaram à redução da resposta insulínica. A razão Ca/Mg, mais que a concentração absoluta de cada íon, mostrou ser um fator regulador primário no processo de secreção de insulina, já que alterações relativamente pequenas resultam em grandes mudanças na secreção total do hormônio (CURRY et al. 1977). No entanto, a menor resposta das células β poderia ser secundária à diminuição do seu conteúdo de potássio, reconhecidamente presente em outros tecidos de ratos submetidos à deficiência de magnésio (GEORGE & HEATON, 1975). Outros efeitos indiretos são relacionados com o aumento da captação de glicose não-insulino e insulino dependentes na deficiência de Mg, reduzindo a necessidade de insulina através de um mecanismo de feedback (McNEILL et al. 1982).

A suplementação de Mg a ratos previamente alimentados com dieta deficiente, evitou a redução da secreção de insulina e a maior tolerância à glicose, mantendo-os em níveis próximos aos dos observados nos animais do grupo controle (REIS et al. **submetido**).

Nos trabalhos *in vitro* onde o Mg foi omitido do meio de incubação os resultados mostraram-se divergentes, ocorrendo substancial redução da secreção de insulina (CURRY

et al.1977) e secreção inalterada, porém com deprimida síntese de insulina (LIN & HAIST, 1973). Tal ligação entre captação de Mg e biossíntese de insulina foi demonstrada por HENQUIN et al. (1986), quando a captação de Mg foi aumentada por agentes que estimulavam a biossíntese e diminuída por aqueles que não a estimulavam.

Na hipótese sobre o controle hormonal da homeostase de Mg, proposto por DURLACH & DURLACH (1984), porém, ocorreria um aumento da secreção de insulina e adrenalina, na tentativa de manter a concentração de Mg celular e, secundariamente, manter o nível de AMPc (adenosina 3', 5'- monofosfato cíclica). Esta indução reativa da secreção de insulina poderia resultar do desaparecimento, durante a deficiência, dos efeitos inibitórios do Mg sobre a secreção de insulina, eventualmente associado à forte estimulação das células β pancreáticas através do aumento do Ca intracelular.

A suplementação de magnésio tem sido preconizada como um tratamento complementar no diabetes mellitus, por trazer possíveis benefícios ao estimular a secreção e sensibilidade à insulina (PAOLISSO et al. 1989, 1994). No entanto, a constatação de que pacientes com DM tipo II sob terapia de longo prazo (3 meses), com altas doses orais de Mg não apresentaram melhora clínica da resistência à insulina (EIBL et al. 1995, VALK et al. 1998), sugere que a deficiência de Mg no diabetes não pode ser tratada simplesmente com o aumento da ingestão desse mineral, ou ainda, que a simples correção dos níveis corpóreos do cátion não sejam suficientes para reverter o quadro de resistência à insulina.

Embora a função bioquímica do Mg no metabolismo da glicose seja reconhecida, evidências sobre o papel da deficiência de Mg na intolerância à glicose permanecem contraditórias. A análise dos efeitos da deficiência de Mg sobre o metabolismo de carboidratos mostra que, de acordo com o modelo experimental, ora predomina o aumento da captação de glicose com consequente redução da glicemia, ora um efeito diabetogênico, com elevação dos níveis glicêmicos. Estas conseqüências parecem resultar do efeito da deficiência de Mg sobre a secreção de hormônios e neuro-hormônios envolvidos na regulação da glicose sangüínea ou sobre o metabolismo periférico. Esta intervenção celular age tanto indiretamente sobre a sensibilidade aos hormônios reguladores e neuro-hormônios, quanto diretamente sobre as reações envolvidas no metabolismo dos carboidratos (DURLACH 1988, DURLACH & DURLACH, 1984, KAHIL 1968). Outros autores

acreditam que a deficiência aguda, com seu efeito redutor do Mg extracelular, e a deficiência celular crônica podem exercer diferentes efeitos sobre o metabolismo da glicose. O Mg extracelular pareceria ser essencial para a manutenção da sensibilidade à insulina, enquanto que a deficiência crônica aceleraria a captação de glicose (LEGRAND et al. 1987).

Portanto, o Mg participa da homeostase da glicose tanto ao regular a secreção da insulina como ao modular sua ação em tecidos alvo. Futuros estudos deverão fornecer informações mais precisas a respeito do papel desse cátion na patogênese do diabetes mellitus e as possíveis implicações terapêuticas da sua reposição ou depleção.

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CAPÍTULO 2

Mg DEFICIENCY IMPAIRS GLUCOSE-INDUCED INSULIN SECRETION AND INCREASES THE SENSITIVITY TO INSULIN IN RATS

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Mg DEFICIENCY IMPAIRS GLUCOSE-INDUCED INSULIN SECRETION AND INCREASES THE SENSITIVITY TO INSULIN IN RATS

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Running head: Glucose homeostasis in Mg deficient rats.

Key words: Mg deficiency, insulin sensitivity, glucose homeostasis, rats.

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Abstract

The effect of Mg deficiency, duration of feeding, and the interaction between these factors on the secretion and action of insulin were investigated. The serum mineral levels, glucose disappearance rate (Kg), and total area under the glucose (ΔG) and insulin (ΔI) curves were compared among rats fed a Mg-deficient diet for 6 (DF-6) or 11 (DF-11) weeks, and rats fed a control diet for the same periods (CO-6 and CO-11 groups). Serum Mg levels were lower in DF-6 and DF-11 rats than in the control groups, but no change in glucose homeostasis was observed among DF-6, CO-6 and CO-11 rats. DF-11 rats showed an elevated Kg and a reduced ΔG compared to CO-11, CO-6 and DF-6 rats, as well as a reduced ΔI compared to the CO-11 and DF-6 groups. For evaluating the effect of supplementation, rats fed a control or Mg-deficient diet for 6 weeks were then fed a Mg-supplemented diet for 5 weeks (SCO and SDF groups, respectively). The serum Mg levels in SDF rats were similar to those in CO-11 and SCO rats, with all of them being significantly higher than in the DF-11 group. SDF rats showed similar Kg, ΔG and ΔI compared to the CO-11 and SCO groups. However, a significantly lower Kg and higher ΔG and ΔI were observed in SDF compared to DF-11 rats. These results indicate that moderate Mg depletion for a long period may increase the sensitivity to insulin, while Mg supplementation in formerly Mg-deficient rats may prevent the increase in sensitivity to this hormone.

Introduction

Numerous studies have demonstrated a major role for magnesium in insulin action and secretion (Bhattacharya, 1961; Gould & Chaudry, 1970; Lin & Haist, 1973; Lostroh & Krahl, 1973; Sanui & Rubin, 1978; Kandeel *et al.* 1996). Direct effects of insulin on Mg metabolism and transport have also been described (Ratzmann, 1985; Gylfe, 1990; Keenan *et al.* 1996). A deficiency in magnesium affects the activities of various enzymes which require magnesium as a cofactor and use high energy phosphate bonds in glucose metabolism (Paolisso *et al.*1990; Elin, 1994). However, comparatively few metabolic alterations have been demonstrated unequivocally in this type of deficiency (Whang *et al.*1969; Gueux & Rayssiguier, 1983; Suárez *et al.*1995; Kimura *et al.*1996) and during supplementation *in vivo* (Gullestad *et al.*1994; Garber *et al.*1995). At least some of the

reported discrepancies in the responses to Mg deficiency may have resulted from the use of animals in which insufficient time was allowed for the effects of Mg deficiency to become uniform (Elin *et al.* 1971; Hunt, 1971).

The present experiments were performed to study the secretion and action of insulin in adult rats fed a Mg-deficient diet for 6 or 11 weeks after weaning and to examine the influence of Mg supplementation on these responses.

Material and methods

Male Wistar rats (21 d old, 40-60 g) bred in the animal facilities of the State University of Campinas were used in all experiments. The rats were housed individually in stainless-steel, wire-bottomed cages in a temperature-controlled room (22 ± 2°) on a 12h light/dark cycle and were fed a commercial stock diet (Purina) for 7 days after arrival. Subsequently, the study was divided in two phases. In the first phase, two groups of rats 4-weeks-old were used: a control group (CO-6) consisting of rats fed a diet containing 507 mg Mg/kg for 6 weeks, and 2) a deficient group (DF-6) consisting of rats fed a diet containing 70 mg Mg/kg for 6 weeks (Table 1). In the second phase, some rats from each of these two groups were divided into two subgroups: 1) those that continued with the original diet for a further 5 weeks, i.e. a total of 11 weeks (CO-11 and DF-11) and 2) those that were repleted by changing to a Mg supplemented diet containing 2100 mg Mg/kg (SCO and SDF).

The rats had free access to deionized water and food throughout the study. Body weight was verified weekly. At the end of each experimental period and after a 12 h fast, blood samples were collected from the abdominal cava vein for the measurement of serum magnesium, calcium and potassium levels. The levels of minerals and the Mg content of the diets were determined by inductively coupled plasma emission spectroscopy (ICP) (Pomeranz & Meloan, 1987) following digestion of the samples in nitric acid.

Intravenous glucose tolerance test (IVGTT)

Glucose (0.75 g/kg body weight) was injected via the caudal vein three days before the end of each experimental period and after a 12 h fast. Plasma glucose and serum insulin concentrations were measured by an oxidase method (Trinder, 1969) and by radioimmunoassay (Scott *et al.* 1981), respectively, in samples obtained from the cut tip of

the tail at 0 (basal) and at 5, 10, 15 and 30 min after injection.. The glucose disappearance rate (Kg) during the IVGTT was calculated using the formula $0.693/t_{1/2}$ and the plasma glucose half-time ($t_{1/2}$) was calculated from the slope of the least square analysis of the plasma glucose concentrations during the linear phase of decline. The glucose and insulin responses during the glucose tolerance test were determined by estimation of the total area under the glucose (ΔG) and insulin (ΔI) curves, using the trapezoidal method (Matthews *et al.*1990).

Statistical analysis

The results are given as the means with their standard deviation for the number of rats indicated. When comparing the changes in body weight for the rats from the first period, Student's non-paired *t* test was used. When the changes in body weight of the second period were compared, Levene's test for the homogeneity of variance, followed by one-way analysis of variance (ANOVA) and the Tukey-Kramer test were used. When comparing the basal serum mineral, glucose and insulin levels, the glucose disappearance rate (Kg), and the total area under the glucose (ΔG) and insulin (ΔI) curves, Levene's test for the homogeneity of variance was initially used. The data were subsequently analyzed by two-way ANOVA, followed by the Tukey-Kramer test for multiple comparisons.

Results

In DF-6 rats, the severity of the classic symptoms of magnesium deficiency varied, with some rats developing ulcerative lesions around the head and neck and hyperemia of the ears. In most DF-6 rats, hyperexcitability was noticed. All the skin lesions disappeared after the initial 6 weeks.

The Table 2 shows that the serum Mg levels of DF-6 rats were significantly lower than in DF-11, and both were lower ($P < 0.001$) than those of CO-6 and CO-11 rats. Two-way ANOVA revealed a significant effect of diet (df 1; F 406.6; $P = 0.000$) and feeding period (df 1; F 7.59; $P = 0.009$), as well as a significant diet x feeding period interaction (df 1; F 33.59; $P = 0.000$). In contrast, the serum Ca levels in DF-6 were significantly higher than in CO-6, CO-11 and DF-11 rats. Again, two-way ANOVA showed a significant effect of diet (df 1; F 5.21; $P = 0.028$), feeding period (df 1; F 6.6; $P = 0.014$) and a diet x

feeding period interaction (df 1; F 32.9; P = 0.000). There were no significant changes in the serum K levels of these groups (Table 2).

At the end of 11th week of treatment, the serum Mg levels of supplemented rats (SDF and SCO groups) were similar to CO-11 rats, and all had serum Mg levels significantly higher than DF-11 rats (Table 3). Two-way ANOVA revealed a significant effect of the previous diet (df 1; F 35.17; P = 0.000), supplementation (df 1; F 61.51; P = 0.000), and a previous diet x supplementation interaction (df 1; F 14.21; P = 0.000). There was no significant change in the serum Ca and K levels of these groups (Table 3).

As shown in Figure 1, the body weight of DF-6 rats was significantly lower ($P<0.05$) than that of CO-6 rats after 3 weeks. However, after 7 weeks of treatment there was no significant difference among rats fed different diets (Figure 2).

The diet (control or Mg-deficient diet), feeding period (6 or 11 weeks) or the interaction between these factors had no significant effect on the fasting plasma glucose and insulin concentrations (Table 2). Similar results were observed for the supplemented rats (SCO and SDF) compared with the non-supplemented groups (CO-11 and DF-11) (Table 3). Thus, neither the Mg-deficient diet and associated feeding period nor Mg-supplementation altered the basal glucose and insulin levels.

The effect of diet and feeding period on Kg during the IVGTT in the control (CO-6 and CO-11) and Mg-deficient groups (DF-6 and DF-11) is shown in Table 2. Two-way ANOVA revealed a significant effect of diet (df 1; F 5.18; P = 0.031), no effect of feeding period, and the presence of a diet x feeding period interaction (df 1; F 5.21; P= 0.031). The longer periods of treatment with the Mg-deficient diet significantly increased the Kg of the DF-11 group relative to the other groups ($P < 0.05$).

When the diets were supplemented with additional Mg, two-way ANOVA showed an effect of the previous diet (df 1; F 4.36; P= 0.047), no effect of supplementation and a supplementation x previous diet interaction (df 1; F 7.7; P= 0.010) on the Kg (Table 3). Mg- supplementation in SDF rats significantly reduced their Kg relative to DF-11 rats ($P<0.02$), however the Kg of SDF was similar to the other groups (Table 3). There was no difference in the Kg of SCO rats when compared with the CO-11 or other two groups.

The ΔG and ΔI during the IVGTT in rats fed for 6 or 11 weeks with control or Mg-deficient diets are shown in Figure 3. Two-way ANOVA showed that diet (df 1; F 4.89; P =

0.036), feeding period (df 1, F 10.23, P = 0.0037) and the interaction between these effects (df 1 ; F 6.97; P = 0.014) had a significant influence on the ΔG. The DF-11 rats had a significantly lower ΔG than CO-6 (P < 0.008), DF-6 (P < 0.002) and CO-11 rats (P < 0.02) (Figure 3A).

Diet had no significant effect on ΔI, whereas the duration of the feeding period (df 1; F 5.27; P = 0.03), and the diet x feeding period interaction (df 1, F 13.21; P = 0.001) affected ΔI (Figure 3B). The greater period of Mg deficiency in DF-11 rats significantly decreased the ΔI relative to DF-6 (P < 0.002) and CO-11 rats (P < 0.02).

Mg supplementation in SDF rats resulted in a higher ΔG than in DF-11 rats (P< 0.02), but no change compared to CO-11 and SCO rats (Figure 4A). The two-way ANOVA revealed a significant effect of previous diet (df 1, F 8.88; P = 0.006), supplementation (df 1, F 4.96, P = 0.035) and a previous diet x supplementation interaction (df 1, F 4.73, P= 0.039) (Figure 4A).

The ΔI of SDF rats was significantly higher than that of DF-11 rats, but similar to other groups (Figure 4B). DF-11 rats showed a reduced ΔI compared to CO-11 rats. Two-way ANOVA revealed a previous diet x supplementation interaction (df 1, F 4.49, P = 0.044). Previous diet and supplementation had no effect on ΔI.

Discussion

Mg deprivation in rats resulted in low serum Mg levels, reduced growth, ulcerative skin lesions and hyperemia of the ears. Although the serum Mg levels remained lower in the Mg deficient rats, the alterations in growth and skin lesions disappeared seven weeks after Mg restriction. Similar finding were reported by Hunt (1971) and Fischer *et al.* (1981). The reduced growth in these rats has been related to the Mg deficiency *per se*, independently of the reduction in food intake (Dϕrup *et al.* 1991; Dϕrup & Clause, 1991), but may also reflect lower levels of serum insulin-like growth factor 1 (IGF-1) (Dϕrup *et al.* 1991). A slower growth rate in Mg deficient animals has been associated with disturbances in mitochondrial energy metabolism, leading to an inhibition of protein synthesis as secondary effect (George & Heaton, 1975). These metabolic disturbances are likely to vary among different tissues and under different conditions, depending on the energy requirements of particular cells (George & Heaton, 1978).

A Mg deficiency for an initial period of 6 weeks did not consistently affect glucose homeostasis. A similar lack of evidence for clear changes in carbohydrate tolerance during Mg depletion was also reported by Whang *et al.* (1969). However, concurrent K and Mg depletion (Whang *et al.* 1969), as well as different durations of Mg depletion (Legrand *et al.* 1987) exert varying effects on glucose metabolism. In the current study, the longer duration of Mg deficiency resulted in improved glucose tolerance in DF-11 rats, as shown by the elevated Kg and low ΔG in the presence of a reduced ΔI. Mg deficiency had no effect on basal glucose and insulin levels in fasted DF-6 and DF-11 rats. Unaltered fasting glucose and insulin levels were also reported by Gueux & Rayssiguier, (1983) and Legrand *et al.* (1987). It is unlikely that an elevation in phosphoenolpyruvate carboxykinase (*EC* 4.1.1.49) activity (McNeill *et al.* 1982), or an increasing non-insulin mediated basal glucose uptake (Kahil *et al.* 1968; Lowney *et al.* 1995) may account for stability.

The significant reduction in ΔG as result of the elevated Kg in DF-11 rats, agrees with the finding that insulin sensitivity may be enhanced by Mg deficiency (Kahil *et al.* 1968; Lowney *et al.* 1995; Kimura *et al.* 1996). Indeed, increased insulin sensitivity of specific tissues has been observed. Divergent results on the tolerance to oral and i.v. glucose loads supposedly reflect the different effects of Mg deficiency on glucose handling by liver and peripheral tissues (Legrand *et al.* 1987). The elevated 2-deoxyglucose (2-DG) transport in diaphragms from Mg-deficient rats (Kahil *et al.*1968) compared to the normal glucose uptake stimulated by insulin and an enhanced hepatic insulin sensitivity (Lowney *et al.*1995) support that observation. A reduced amount and activity of GLUT 4 (insulin-responsive glucose transporter) in adipose tissue, together with low plasma insulin levels, without hyperglycemia, were observed in Mg-deficient rats (Kimura *et al.*1996), a finding that agrees with the differing insulin sensitivities of specific tissues.

The reduced Kg and consequently elevated ΔG and ΔI seen in SDF rats compared to DF-11 rats, showed that Mg supplementation prevent the increase in glucose tolerance in the former rats compared to the latter. In addition, Mg supplementation did not change glucose tolerance in SCO or SDF rats compared to CO-11 rats. Interestingly, an elevated uptake of 2-DG in the diaphragm of Mg-deficient rats was promptly reversed by the addition of Mg to the incubation medium (Kahil *et al.*1968). However, in clinical situations where a diminished glucose tolerance was attributed to a Mg deficiency, supplementation

with Mg either improved (Paolisso *et al.* 1989, 1994) or did not change the insulin secretion and sensitivity (Eibl *et al.* 1995).

A poor insulin response to glucose load frequently accompanies a Mg deficiency (Gueux & Rayssiguier, 1983; Legrand *et al.* 1987). The mechanism responsible for the altered insulin levels remains unclear. Previous reports have suggested changes in insulin biosynthesis and the omission of extracellular Mg inhibits glucose stimulation of insulin biosynthesis in isolated rat islets (Lin & Haist, 1973) by mechanisms linked to the ability of glucose and other stimulators of insulin synthesis to increase Mg uptake (Henquin *et al.* 1983, 1986). The pancreas of Mg-deficient rats is reported to have a low insulin content (Legrand *et al.* 1987).

The effect of Mg on stimulated insulin release is not clear. Mg omission inhibits insulin secretion (Curry *et al.* 1977), while reduced Mg levels either increase (Bennett *et al.* 1969) or have no effect on insulin secretion (Lin & Haist, 1973; Lowney *et al.* 1995). These divergent results may reflect possible imbalances in the optimal ratio between Ca and Mg necessary for normal insulin secretion (Atwater *et al.* 1983; Panzig *et al.* 1985). Some studies have shown that the duration of Mg deficiency and the Ca content of the experimental diet are important determinants of the animal's ability to maintain Ca homeostasis during Mg depletion (Anast & Gardner, 1985; Welsh & Weaver, 1988).

Previous reports have described the possible mechanism by which Mg participates in the regulation of insulin secretion in RIN m5F cells. The intracellular concentration of Mg was shown to be dependent upon extracellular Mg, with the influx occuring through voltage-dependent Ca channels. Extracellular Mg may competitively inhibit voltage-dependent Ca channels and inhibit Ca currents leading to decreased insulin secretion. In the absence of Mg in the extracellular space, this inhibition would not occur, resulting in enhanced insulin secretion (Murakami *et al.* 1992; Ishizuka *et al.* 1994). In contrast, a Mg deficiency in rats is accompained by changes in the cellular distribution of calcium and loss of cellular potassium (George & Heaton, 1975) which could contribute to a reduction in insulin release.

The present results show that Mg deficiency is characterized initially by a reduction in body weight gain during the first six weeks of treatment followed by a return to normal growth from the 6th to 11th week. Interestingly, the restoration of body weight gain in DF-

11 rats did not result in a defective sensitivity to insulin, suggesting that Mg deficiency produced a higher sensitivity to insulin despite the increased weight.

In conclusion, our results suggest that a moderate Mg deficiency for 11 weeks resulted in an elevated glucose tolerance despite the reduced insulin response to glucose infusion. In contrast, Mg supplementation in SDF and SCO rats did not change their glucose tolerance compared to CO-11 rats, but prevented an increase in insulin sensivity, as seen in DF-11 rats. There was no change in the glucose tolerance of rats fed a Mg-deficient diet for 6 weeks, indicating that in cases of less severe Mg deprivation, a greater period would be necessary to produce alterations in glucose metabolism.

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Table 1. Composition of the control diet.

Ingredients	g/kg
Cornstarch	397.5
Casein (85% protein)	200.0
Dextrinized cornstarch	132.0
Sucrose	100.0
Soybean oil	70.0
Fiber	50.0
Mineral mix (AIN-93)*	35.0
Vitamin mix (AIN-93)*	10.0
L-Cystine	3.0
Choline bitartrate	2.5

*Detailed composition given by Reeves *et al.* (1993).
The composition of the experimental diets was the same as the control diet, except for the low level of Mg^{2+} (70 mg of Mg/kg) in the Mg deficient diet, the addition of $MgSO_4.7H_2O$ to the Mg supplemented diet to bring the Mg^{2+} levels to 2100 mg/kg.

Table 2. Effect of diet, feeding period and the diet x feeding period interaction on serum Mg, Ca and K levels, fasting plasma glucose and insulin concentrations and glucose disappearance rate (Kg) in rats fed control (CO-6 and CO-11) or Mg-deficient (DF-6 and DF-11) diets for 6 or 11 weeks, respectively. (Mean values and standard deviations for the no. of rats shown in parentheses).

Group	Mg (mmol/l) ** Mean SD	Ca (mmol/l) † Mean SD	K (mmol/l) Mean SD	Glucose (mmol/l) Mean SD	Insulin (nmol/l) Mean SD	Kg (%/min) * Mean SD
CO-6	0.93 0.06[a] (12)	2.0 0.17[b] (12)	3.02 0.32 (12)	6.0 0.90 (6)	0.30 0.12 (6)	1.9 0.60[b] (6)
CO-11	0.85 0.15[a] (8)	2.2 0.10[b] (8)	3.47 0.21 (8)	5.2 0.75 (6)	0.24 0.06 (6)	1.8 0.35[b] (6)
DF-6	0.30 0.04[c] (12)	2.4 0.32[a] (12)	3.34 0.35 (12)	6.2 0.90 (10)	0.27 0.08 (10)	1.9 0.72[b] (10)
DF-11	0.46 0.05[b] (10)	2.0 0.15[b] (10)	3.40 0.35 (10)	5.2 0.50 (7)	0.20 0.07 (7)	3.2 1.22[a] (7)
Diet ‡	P = 0.000	P = 0.028	NS	NS	NS	P = 0.031
Feeding period	P = 0.009	P = 0.014	NS	NS	NS	NS
Diet x Feeding period	P = 0.000	P = 0.000	NS	NS	NS	P =0.031

Values with different superscript letters are significantly different (**P < 0.001, †P < 0.01, *P < 0.05). ‡Overall P values for differences in diet, feeding period and the diet x feeding period interaction; NS = not significant.

Table 3. Effect of previous diet, supplementation and the previous diet x supplementation interaction on serum Mg, Ca and K levels, fasting plasma glucose and insulin concentrations and glucose disappearance rate (Kg) in rats fed control or Mg-deficient diets for 11 weeks (CO-11 and DF-11, respectively), or in rats fed control or Mg-deficient diets for 6 weeks and then changed to a Mg-supplemented diet for 5 weeks (SCO and SDF, respectively). (Mean values and standard deviations for the no. of rats shown in parentheses).

Group	Mg (mmol/l) **	Ca (mmol/l)	K (mmol/l)	Glucose (mmol/l)	Insulin (nmol/l)	Kg (%/min) †
	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD
CO-11	0.85 0.15[a] (8)	2.2 0.10 (8)	3.47 0.21 (8)	5.2 0.75 (6)	0.24 0.06 (6)	1.8 0.35[b] (6)
SCO	1.00 0.09[a] (5)	2.3 0.10 (5)	3.33 0.24 (5)	6.4 0.52 (5)	0.23 0.10 (5)	2.0 0.48a[b] (5)
DF-11	0.46 0.05[b] (10)	2.0 0.15 (10)	3.40 0.35 (10)	5.2 0.50 (7)	0.20 0.07 (7)	3.2 1.22[a] (7)
SDF	0.91 0.11[a] (10)	2.3 0.11 (10)	3.55 0.27 (10)	5.7 0.89 (9)	0.22 0.05 (9)	1.9 0.42[b] (9)
Previous diet‡	P = 0.000	P = 0.031	NS	NS	NS	NS
Supplementation	P = 0.000	P = 0.000	NS	NS	NS	P = 0.047
Previous diet x suppl.	P = 0.000	NS	NS	NS	NS	P = 0.01

Values with different superscript letters are significantly different (**P < 0.001, †P < 0.02). ‡Overall P values for differences in previous diet, supplementation and the previous diet x supplementation interaction; NS = not significant.

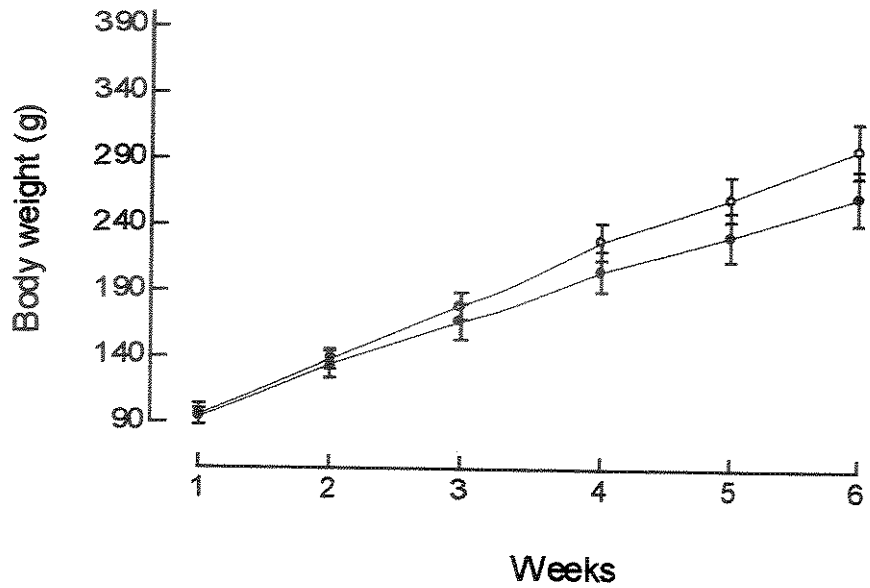

Figure 1. Effect of Mg deficiency on the body weight of rats. Mean values and standard deviations are represented by vertical bars for twelve control (CO-6, open circles) and twenty-two Mg deficient (DF-6, closed circles) rats. The body weights of CO-6 and DF-6 were significantly different ($P < 0.05$) at all points except in the first two weeks.

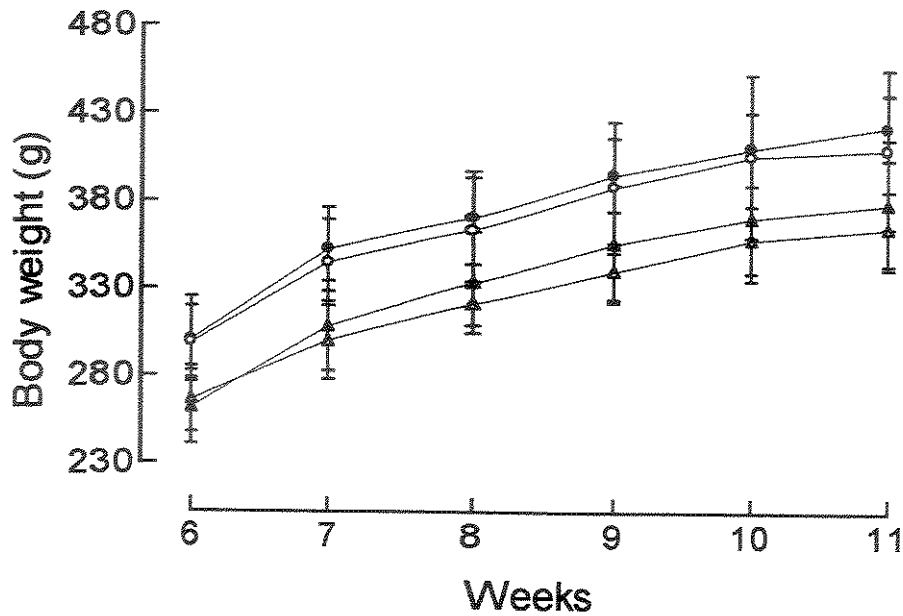

Figure 2. Effect of Mg deficiency and supplementation on the body weight of rats. Mean values and standard deviations are represented by vertical bars for eight or ten rats fed for eleven weeks with a control (CO-11, open circles) or Mg-deficient (DF-11, open triangles) diets, respectively, and five control (SCO, closed circles) or ten Mg-deficient (SDF, closed triangles) rats fed a Mg-supplemented diet for five weeks. The body weights of the four groups were significantly different ($P < 0.05$) only at weeks 6 and 7.

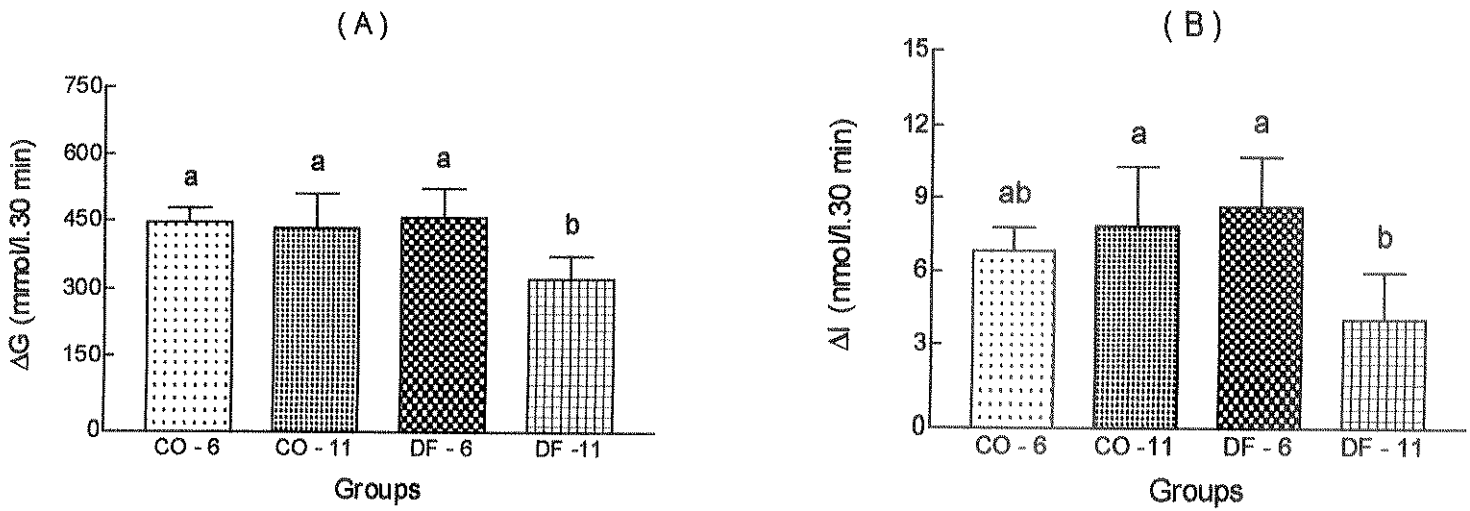

Figure 3. Total areas under the glucose (ΔG) and insulin (ΔI) curves (A and B, respectively) obtained by the intravenous glucose tolerance test in rats maintained on control (CO-6 and CO-11) or Mg-deficient (DF-6 and DF-11) diets for 6 or 11 weeks, respectively. Mean values and standard deviations are represented by vertical bars for six to ten rats. Mean values with different superscript letters are significantly different ($P < 0.02$).

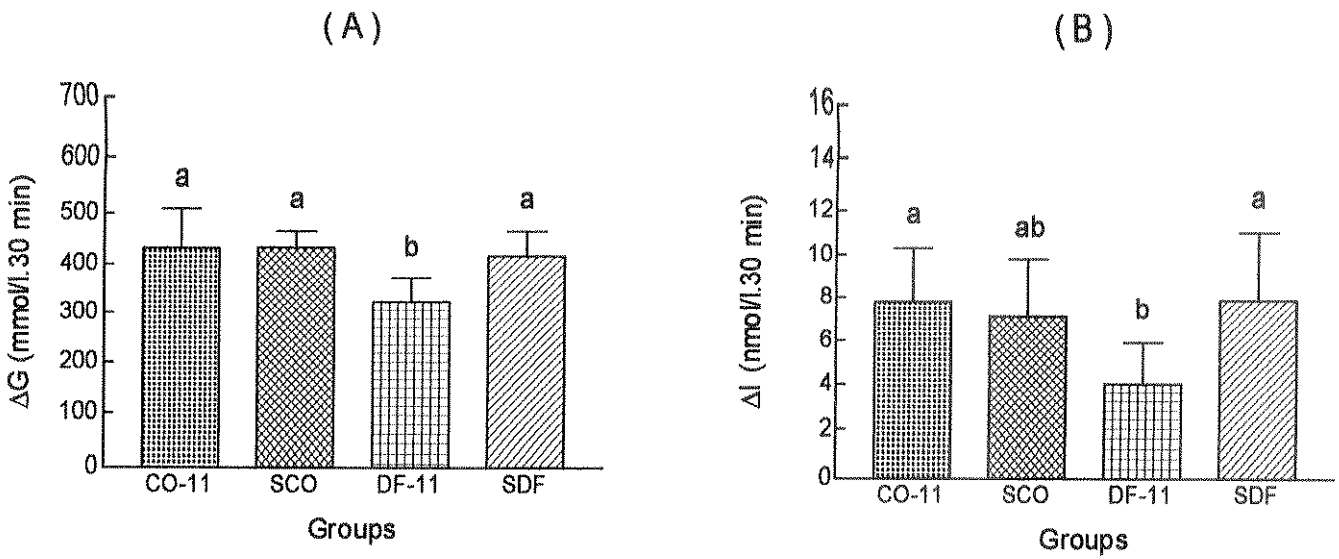

Figure 4. Total areas under the glucose (ΔG) and insulin (ΔI) curves (A and B, respectively) obtained by the intravenous glucose tolerance test in rats maintained on control or Mg-deficient diets for 11 weeks (CO-11 and DF-11, respectively) or in rats Mg repleted by feeding a Mg-supplemented diet from the 6th to 11th week (SCO and SDF, respectively). Values are means with their standard deviation represented by vertical bars for five to nine rats. Mean values with different superscript letters are significantly different ($P < 0.02$).

CAPÍTULO 3

MAGNESIUM DEFICIENCY MODULATES THE INSULIN SIGNALING PATHWAY IN LIVER BUT NOT IN MUSCLE OF RATS

TRABALHO ACEITO PARA PUBLICAÇÃO NO THE JOURNAL OF NUTRITION

Magnesium Deficiency Modulates the Insulin Signaling Pathway in Liver but Not Muscle of Rats[1]

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ABSTRACT Altered insulin secretion and sensitivity have been observed in Mg-deficient animals. However, the effects of Mg deficiency and supplementation on intracellular signaling events triggered by insulin are unknown. Therefore, we studied the early steps of insulin action in muscle and liver of rats fed Mg-deficient (DF-6, DF-11) or control (CO-6, CO-11) diets for 6 or 11 wk, respectively, and Mg-deficient or control diets for 6 wk, followed by Mg supplementation for 5 wk (SDF and SCO groups, respectively). There were no differences in the glucose disappearance rate (K_{itt}) or insulin signaling between CO-6 and DF-6 rats. Between the two groups of rats fed for 11 wk, the DF-11 group had a significantly greater K_{itt}. SDF and SCO rats had K_{itt} that did not differ from CO-11 rats, but that were significantly lower than in DF-11 rats. In the latter rats, insulin receptor and insulin receptor substrate-1 protein and phosphorylation levels were elevated in liver and there was a greater association between the insulin receptor substrate-1 and p85 subunit of phosphatidylinositol 3-kinase compared with CO-11 rats. There were no differences in the early steps of insulin action in SDF and control rats. These results suggest that the normal insulin sensitivity maintained by Mg supplementation and the increased insulin sensitivity produced by a long period of Mg deprivation may result, at least in part, from alterations in or maintenance of the early molecular steps of insulin action in hepatic tissue. J. Nutr. 130: 133–138, 2000.

KEY WORDS: • *insulin receptor* • *insulin receptor substrate-1* • *magnesium deficiency* • *magnesium supplementation* • *rats*

Magnesium deficiency is frequently associated with changes in carbohydrate homeostasis (Gueux and Rayssiguier 1983, Kimura et al. 1996, Lowney et al. 1995). Studies in diabetic patients have attempted to correlate hypomagnesemia and reduced erythrocyte Mg concentration with poor glycemic control and the development of complications (Bloomgarden 1995, Paolisso et al. 1990, Tosiello 1996). Because a low Mg concentration may be a consequence or a cause of insulin resistance (Paolisso et al.1990), it may be premature to assign a primary role to Mg in abnormal carbohydrate metabolism (Garber et al. 1992).

We demonstrated recently that rats fed a Mg-deficient diet for a long period showed increased insulin sensitivity, accompanied by a reduction in insulin secretion (unpublished data). However, the molecular mechanism responsible for the change was not established.

Insulin action in target tissues is mediated by the heterotetrameric insulin receptor (IR)[3]. After ligand binding, the receptor tyrosine kinase domain is activated, resulting in receptor autophosphorylation and tyrosine phosphorylation of several intermediate proteins, including insulin receptor substrate 1 (IRS-1), a cytoplasmic protein with an apparent molecular weight of 160–185 kDa (Cheatam and Kahn 1995, Myers and White 1996). Tyrosine phosphorylated IRS-1 then couples the insulin receptor to downstream signaling pathways by acting as a docking protein for the src homology-2 (SH2) domain–containing proteins. SH2 proteins are the link between upstream tyrosine kinases and downstream signaling elements. One of the substrates of tyrosine phosphorylated IRS proteins is the lipid metabolizing enzyme phosphatidylinositol (PI) 3-kinase (Kelly and Ruderman 1993). In addition to its

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[3] Abbreviations used: CO, control group; DF, Mg-deficient group; ICP, inductively coupled plasma emission spectroscopy; IR, insulin receptor; IRS-1, insulin receptor substrate-1; ITT, insulin-tolerance test; ivITT, intravenous insulin-tolerance test; K_{itt}, rate constant for serum glucose disappearance during insulin-tolerance test; PI 3-kinase, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonylfluoride; SCO, Mg-supplemented control group; SDF, Mg-supplemented deficient group; SH2, src homology-2.

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TABLE 1

Composition of the control, Mg-deficient and Mg-supplemented diets

Ingredient	g/kg diet
Cornstarch	397.5
Casein (85% protein)	200.0
Dextrinized cornstarch	132.0
Sucrose	100.0
Soybean oil	70.0
Cellulose	50.0
Mineral mix (AIN-93)[1,2]	35.0
Vitamin mix (AIN-93)[1]	10.0
L-Cystine	3.0
Choline bitartrate	2.5

[1] Detailed composition given by Reeves et al. (1993).

[2] A similar composition was used in the control, Mg-deficient and Mg-supplemented diets, except for the addition of $MgSO_4 \cdot 7H_2O$ to provide (per kg) 507 mg of Mg in the control diet, 70 mg of Mg in the Mg-deficient and 2100 mg of Mg in the Mg-supplemented diet.

role in the regulation of mitogenesis, cellular transformation, differentiation, chemotaxis and membrane ruffling (Lange et al. 1998), activated PI 3-kinase is involved in insulin-stimulated glucose uptake and glycogen synthesis (Holman and Kasuga 1997). Thus, the pathway involving the insulin receptor, IRS-1 proteins and PI 3-kinase plays some role in glucose clearance.

In this study, we investigated the phosphorylation state of IR and IRS-1, as well as the association of the latter with PI 3-kinase in the liver and muscle of Mg-deficient and Mg-supplemented rats treated acutely with insulin.

MATERIALS AND METHODS

Antibodies and chemicals. Monoclonal anti-phosphotyrosine antibody and anti-PI 3-kinase (p85) antibody were from Santa Cruz (Santa Cruz, CA). The anti-IRS-1 and anti-IR antisera were previously described (Sun et al. 1992). [^{125}I] Protein A was from Amersham (Buckinghamshire, UK) and Protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 mm) was from Schleicher & Schuell (Keene, NH) and the chemicals were from Sigma Chemical (St. Louis, MO).

Buffers. Buffer A consisted of 100 mmol/L Tris, 10 g/L SDS, 50 mmol/L (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (pH 7.4), 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L ethylenediaminetetraacetic acid and 10 mmol/L sodium vanadate. Buffer B was similar to buffer A except that 10 g/L of Triton X-100 replaced 10 g/L of SDS, and 2 mmol/L phenylmethylsulfonylfluoride (PMSF) and 0.1 g aprotinin/L were added. Buffer C contained 100 mmol/L Tris, 10 mmol/L sodium vanadate, 10 mmol/L EDTA and 10 g/L Triton X-100.

Animals. Male Wistar rats (21 d old, 40–60 g), bred at the State University of Campinas animal breeding center, were allowed 1 wk to adapt to housing conditions during which they were fed a nonpurified diet (Purina laboratory diet, Paulinia, São Paulo, Brazil). Thereafter, the rats were fed either a control diet (CO-6 group) containing 507 mg Mg/kg or a Mg-deficient diet (DF-6 group) containing 70 mg Mg/kg (**Table 1**). After 6 wk of treatment, some rats from the CO-6 and DF-6 groups (referred to as CO-11 and DF-11) were maintained on their respective diets for an additional 5 wk (**Fig. 1**). The remaining rats from both groups were changed to a Mg-supplemented diet containing 2100 mg Mg/kg (Table 1) for the same period (referred to as supplemented control, SCO, and supplemented deficient, SDF groups). The rats had free access to deionized water and food throughout the study. They were housed individually, and rooms were maintained at 21 ± 2°C on a 12-h light:dark cycle (lights on from 0600 to 1800 h). Food deprivation began at 1800 h. The rats were weighed at the end of the first and second phases. At the end of each experimental period and after 12 h of food deprivation, blood samples were collected from the abdominal cava vein, allowed to clot and the sera stored at −20°C for the subsequent measurement of insulin by RIA (Scott et al. 1981) and for the measurement of magnesium and calcium. The minerals in the sera and diets were measured by inductively coupled plasma emission spectroscopy (ICP) using an argon plasma emission spectrophotometer (BAIRD ICP 2000; Baird , Deford, MA). Mg and Ca serum levels were measured after digestion of the samples in 10 mol/L HNO_3 and appropriate dilution with deionized water (Slavin et al. 1975). Preparatory to analysis, diets were dried, calcinated at 450°C and diluted in 0.8 mol/L HNO_3 (AOAC 1997). All experiments involving animals were approved by the State University of Campinas Ethics Committee (São Paulo, Brazil).

Insulin-tolerance test. An intravenous insulin-tolerance test (ivITT) was performed in both phases. After 12 h of food deprivation, insulin (0.5 mL of a 10^{-5} mol/L solution) was injected intravenously, and samples for plasma glucose determination were collected from the cut tip of the tail at 0 (basal), 4, 8,12 and 16 min after hormone injection. Plasma glucose levels were determined by the glucose oxidase method. The glucose disappearance rate (K_{itt}) during the ivITT was calculated using the formula $0.693/t_{1/2}$ (Lundbæk 1962). The plasma glucose half-time ($t_{1/2}$) was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase. On the basis of data from Bonora et al. (1989), a high degree of correlation between the insulin-tolerance test (ITT) and clamp studies confirmed the reliability of this study.

Tissue extraction, immunoblotting and immunoprecipitation. The rats were anesthetized with sodium amobarbital (15 mg/kg body weight, intraperitoneally) and used 10–15 min later, as soon as anesthesia was ensured by loss of the pedal and corneal reflexes. After the portal vein was exposed, a bolus injection of 0.5 mL of normal saline (9 g/L NaCl), with or without insulin (10^{-5} mol/L) was given. The insulin dose was chosen on the basis of previous work in which the amount of insulin required to achieve a large signal was deter-

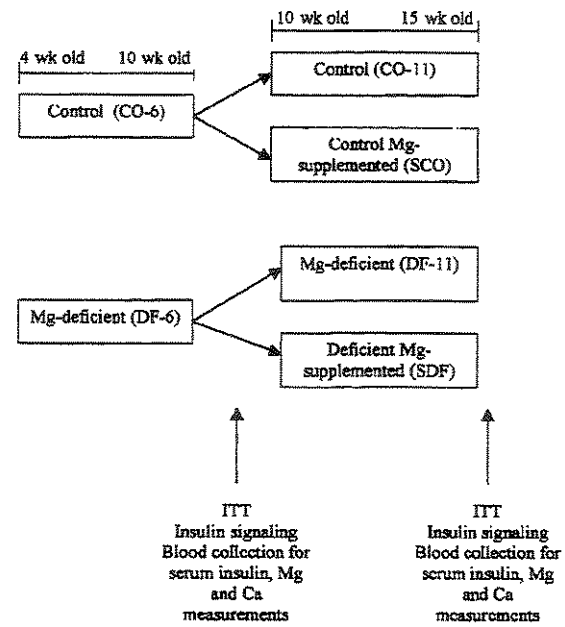

FIGURE 1 The experimental protocol used in this study of the early effects of insulin action in muscle and liver of rats fed deficient (DF-6, DF-11) or control (CO-6, CO-11) diets for 6 or 11 wk, respectively, and Mg-deficient or control diets for 6 wk, followed by Mg supplementation for 5 wk (SDF and SCO groups, respectively). Abbreviation: ITT, insulin-tolerance test.

mined (Saad et al. 1995). At 30 s postinjection, the bolus injection of insulin leads to a transient rise in the peripheral concentration of this hormone that is five to ten times the postprandial levels. The very high levels of insulin attained suggest that different levels of circulating insulin cannot explain some of the variations observed. A fragment of the liver was excised 30 s later, minced coarsely and immediately homogenized in freshly prepared boiling buffer A for immunoblotting, or freshly prepared ice-cold buffer B for immunoprecipitation. Approximately 90 s after injection, hind-limb muscle (musculus gastrocnemius) was quickly excised and homogenized as described for liver. The insoluble material in both extracts was removed by centrifugation for 45 min at 50,000 × *g* at 4°C. The protein concentration in the supernatants was determined by the Bradford method (Bradford 1976).

For immunoblotting, samples of 150 μg of total protein were suspended in 50 μL of Laemmili sample buffer, boiled for 5 min and subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad (Hercules, CA) miniature slab gel apparatus (Mini-Protean). For immunoprecipitation, samples containing 3 mg of total protein were incubated with 15 μL of anti-IRS-1 or anti-IR antiserum at 4°C overnight. The immune complexes were precipitated for 1 h with protein A-Sepharose 6 MB and were repeatedly washed in buffer C (five times). The pellets were resuspended in 50 μL of Laemmli sample buffer and boiled for 5 min before loading onto the gel. Electrotransfer of proteins from the gel to nitrocellulose was performed as described by Towbin et al. (1979). The membranes were blocked, probed and developed as described previously (Saad et al. 1995).

Statistics. The results are presented as means ± SD for the number of rats (*n*) indicated. Student's *t* test for unpaired data was used for direct comparisons between the CO-6 and DF-6 groups. Levene's test followed by one-way or two-way ANOVA and the Tukey-Kramer test for multiple comparisons among groups were used in the second phase. *P*-values < 0.05 were considered to indicate significant differences.

RESULTS

Characteristics of the rats. After 6 wk, the serum Mg concentration in DF-6 rats (0.4 ± 0.02 mmol/L) was significantly lower ($P < 0.001$) than that in CO-6 rats (1.05 ± 0.2 mmol/L), but there was no difference in the serum Ca concentrations of the two groups. By wk 11 of treatment, the serum Mg concentrations of DF-11 rats were significantly ($P < 0.01$) lower than those of CO-11, SDF and SCO rats (Table 2). Two-way ANOVA revealed a significant effect of the previous diet (df 1; F 25; $P < 0.001$) and supplementation (df 1; F 47; $P < 0.001$), as well as a two-way interaction between the previous diet and supplementation (df 1; F 85; $P < 0.001$) (Table 2). The serum Ca concentrations during the second phase did not differ among the groups. A significant effect of the previous diet (df 1; F 7; $P = 0.03$) and supplementation (df 1; F 29.7; $P = 0.005$) was observed, but with no significant two-way interaction (Table 2). The classic signs of Mg deficiency were observed from wk 3 to 5 of treatment, after which hyperemia of the ears and ulcerative lesions around the head and neck disappeared. During the first phase, the body weight of DF-6 rats was 5% lower than that of CO-6 rats (DF-6, 294.4 ± 17.8 g, $n = 13$, and CO-6, 310 ± 17.6 g, $n = 12$, $P < 0.05$). However, at the end of the second phase, the body weights did not differ among the groups (CO-11 rats, 413 ± 36.7 g, $n = 6$; DF-11 rats 385 ± 20.4 g, $n = 7$; SDF rats, 399.5 ± 35.3 g, $n = 6$; SCO rats, 413.6 ± 17.5 g, $n = 6$).

ITT. There were no significant differences in the K_{itt} and plasma glucose and serum insulin concentrations of food-deprived rats from the first phase (data not shown). By wk 11 of treatment, rats in the second phase had basal plasma glucose and serum insulin concentrations that did not differ, and two-way ANOVA showed no significant main effect or interaction (data not shown). In contrast, the K_{itt} of DF-11 rats was significantly higher ($P < 0.05$) than that of the other groups (Table 2). Two-way ANOVA showed a significant main effect of the previous diet (df 1; F 4.4; $P = 0.047$) and supplementation (df 1; F 8.2; $P = 0.008$), as well as a two-way interaction between the previous diet and supplementation (df 1; F 8.24; $P = 0.008$).

TABLE 2

Glucose disappearance rates (K_{itt}) and basal serum Mg and Ca concentrations in control (CO-11) and Mg-deficient rats (DF-11) fed their respective diets for 11 wk, and in Mg-deficient supplemented (SDF) and control supplemented rats (SCO) fed a Mg-deficient or control diet for 6 wk and then changed to a Mg-supplemented diet for 5 wk[1,2]

Group	K_{itt}	Mg	Ca
	%/min	mmol/L	
CO-11	2.5 ± 0.65[b]	1.2 ± 0.38[a]	2.1 ± 0.30
n	8	7	7
DF-11	4.0 ± 1.50[a]	0.5 ± 0.08[b]	2.1 ± 0.35
n	4	8	8
SDF	2.0 ± 1.10[b]	1.1 ± 0.23[a]	2.2 ± 0.21
n	7	6	6
SCO	2.5 ± 0.75[b]	1.1 ± 0.10[a]	2.3 ± 0.10
n	8	6	6

[1] Values are the means ± SD; *n* = number of rats.
[2] Different letters in a column indicate significant differences, ($P < 0.05$).

Effect of a Mg-deficient diet on IR and IRS-1 protein and phosphorylation levels and IRS-1/PI 3-kinase association in rat liver and muscle. The protein levels of IR and IRS-1 and the rate of IR and IRS-1 phosphorylation in response to insulin, as well as the rate of IRS-1/p85/PI 3-kinase association, did not differ between the CO-6 and DF-6 groups in either tissue studied (data not shown).

Effect of Mg deficiency and supplementation on IR and IRS-1 protein and phosphorylation levels and IRS-1/PI 3-kinase association in rat muscle. Mg deficiency and subsequent supplementation had no effect on the early steps of insulin signaling in muscle from CO-11, DF-11 and SDF rats. There were no differences in the amount of IR protein (100 ± 23%, $n = 8$ for CO-11; 85 ± 51%, $n = 7$ for DF-11, and 74 ± 36%, $n = 7$ for SDF), or IRS-1 protein (100 ± 16.5%, $n = 9$ for CO-11, 121 ± 15%, $n = 5$ for DF-11, and 125 ± 24%, $n = 7$ for SDF) among rats in the second phase. Similarly, there were no significant differences in the insulin-stimulated phosphorylation of IR (100 ± 24%, $n = 5$ for CO-11, 84 ± 24%, $n = 4$ for DF-11, and 94 ± 15%, $n = 4$ for SDF), and IRS-1 (100 ± 48%, $n = 8$ for CO-11, 131 ± 31%, $n = 5$ for DF-11, and 118 ± 25%, $n = 14$ for SDF), or in the IRS-1/p85/PI 3-kinase association (100 ± 31%, $n = 8$ for CO-11, 93 ± 18.5%, $n = 4$ for DF-11, and 124 ± 21%, $n = 12$ for SDF).

Effect of Mg deficiency and supplementation on IR and IRS-1 protein and phosphorylation levels and IRS-1/PI 3-kinase association in rat liver. Figure 2A shows the IR protein levels in the liver of second-phase rats as detected by immunoblotting. Higher IR levels were observed in DF-11 than in CO-11 rats ($P < 0.001$). In SDF rats, the IR level was not different from that of CO-11 rats, but was significantly lower than that in DF-11 rats ($P < 0.005$).

Using a specific antipeptide antibody against IRS-1 (Fig.

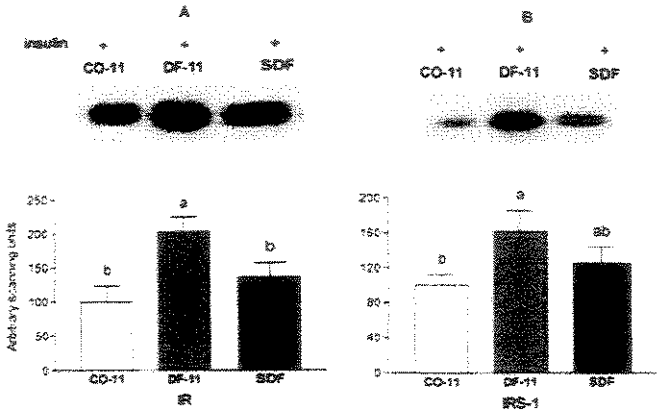

FIGURE 2 Fluorographs obtained after SDS-PAGE of total extracts of liver from control (CO-11) (Fig. 2*A*, $n = 9$; Fig. 2*B*, $n = 7$), Mg-deficient (DF-11) (Fig. 2*A*, $n = 6$; Fig. 2*B*, $n = 6$) and Mg-supplemented (SDF) (Fig. 2*A*, $n = 9$; Fig. 2*B*, $n = 7$) rats. The rats were injected with saline (not shown) or insulin; 30 s later, liver was excised and homogenized with extraction buffer A at 100°C as described in the Materials and Methods section. After centrifugation, aliquots of supernatants containing equal amounts of protein were resolved by SDS-PAGE on 6% polyacrylamide gels, transferred to nitrocellulose, analyzed using (*A*) anti-insulin receptor or (*B*) anti-insulin receptor substrate-1 antibodies, in conjunction with [^{125}I] protein A, and then subjected to autoradiography. The means ± SD (n = 6–9) of arbitrary scanning units are depicted at the bottom of the figure. Different letters indicate significant differences, $P < 0.05$.

2*B*), the level of this protein was 62.5 ± 24% greater in the liver of DF-11 rats ($P < 0.002$) compared with CO-11 rats. In the SDF group, the IRS-1 levels were not significantly different from those in either CO-11 or DF-11 rats.

Liver samples previously immunoprecipitated with anti-insulin receptor antibody and immunoblotted with antiphosphotyrosine antibody (**Fig. 3***A*) showed a 88.5 ± 26% greater insulin-stimulated IR phosphorylation in DF-11 rats compared with CO-11 rats ($P < 0.02$), whereas in SDF rats, the values were not significantly different from those of the other two groups.

Immunoprecipitation with anti-IRS-1 antibodies and immunoblotting with antiphosphotyrosine antibodies showed that the insulin-stimulated phosphorylation of IRS-1 was 63 ± 28% greater ($P < 0.01$) in the liver of DF-11 rats compared with CO-11 rats, whereas in SDF rats, the increase in insulin-stimulated IRS-1 phosphorylation was not different from the other groups (Fig. 3*B*).

To examine the association of the 85-kDa subunit of PI 3-kinase with IRS-1, blots of samples that had been previously immunoprecipitated with anti-IRS-1 antibodies were incubated with anti-PI 3-kinase antibody (**Fig. 4**). As expected, a greater (41 ± 22%) insulin-stimulated IRS-1-p85/PI 3-kinase association was detected in the liver of DF-11 rats compared with CO-11 rats. SDF rats tended ($P = 0.195$) to have a greater (18.5 ± 10%) IRS-1-p85/PI 3-kinase association than CO-11 rats.

DISCUSSION

In humans and in animal models, Mg deficiency modulates insulin sensitivity, and may or may not be associated with impaired insulin secretion (Lowney et al. 1995, Nadler et al. 1993). Our results demonstrate that insulin sensitivity, measured by the glucose disappearance rate (K_{itt}) during the 15 min ITT, was not different in DF-6 rats and CO-6 rats. In addition, DF-6 rats had a normal glucose uptake at maximal insulin concentration, as previously described (Suárez et al. 1995). On the other hand, after an additional period of deficiency, DF-11 rats showed an improvement in insulin sensitivity, as revealed by the elevated K_{itt} compared with the CO-11, SDF and SCO groups, suggesting an increased glucose uptake during Mg deficiency. The mechanism underlying the improvement in insulin sensitivity induced by Mg deficiency is not fully understood. The increased glucose disposal observed in Mg-deficient rats has been associated with a noninsulin-mediated glucose uptake and an improvement in hepatic insulin sensitivity (Lowney et al. 1995).

The Mg-deficient diet fed to DF-6 rats did not affect the insulin sensitivity or the amount and action of the proteins involved in the early steps of insulin action in the two tissues studied. However, a longer Mg deficiency significantly increased the insulin sensitivity and the insulin-induced IR and IRS-1 protein and tyrosine phosphorylation levels in the liver of DF-11 rats. The increased phosphorylation of IRS-1 was accompanied by an increase in IRS-1/PI 3-kinase association in this tissue. These increases in IR and IRS-1 phosphorylation were observed in the liver, but not in muscle, suggesting a tissue-specific effect of Mg deficiency. Our results may be of biological importance because an increase in insulin receptor and IRS-1 phosphorylation, as well as IRS-1/PI 3-kinase association, has been associated with enhanced insulin sensitivity in animal models of insulin resistance (Carvalho et al. 1997) and in animal models of protein malnutrition (Latorraca et al. 1998, Reis et al. 1997).

Food-deprived and streptozotocin-induced diabetic animals show an increase in total receptor amount, in IRS-1 phosphor-

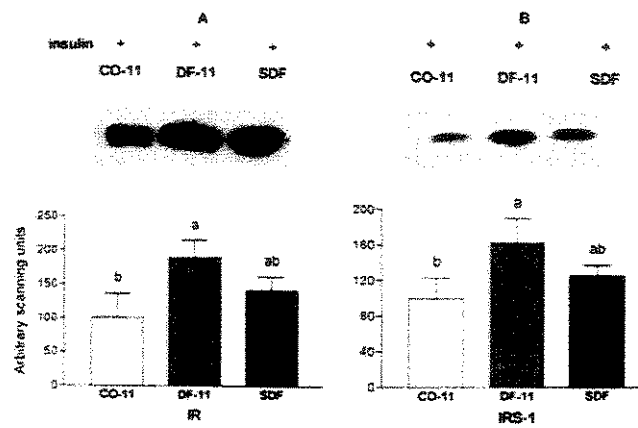

FIGURE 3 Fluorograph obtained after SDS-PAGE of immunoprecipitates from liver from control (CO-11) (Fig. 3*A*, $n = 6$; Fig. 3*B*, $n = 8$), Mg-deficient (DF-11) (Fig. 3*A*, $n = 4$; Fig. 3*B*, $n = 4$) and Mg-supplemented (SDF) (Fig. 3*A*, $n = 9$; Fig. 3*B*, $n = 6$) rats. The rats were injected with saline (not shown) or insulin; 30 s later, liver was excised and homogenized in ice-cold extraction buffer B as described in the Materials and Methods section. After centrifugation, aliquots of the supernatants containing equal amounts of protein were immunoprecipitated using (*A*) anti-insulin receptor or (*B*) anti-insulin-receptor substrate-1 antibodies and then resolved by SDS-PAGE on 6% polyacrylamide gels. The nitrocellulose transfers were blotted using antiphosphotyrosine antibody in conjunction with [^{125}I] protein A, and then subjected to autoradiography. The means ± SD (n = 4–9) of arbitrary scanning units are depicted at the bottom of the figure. Different letters indicate significant differences, $P < 0.05$.

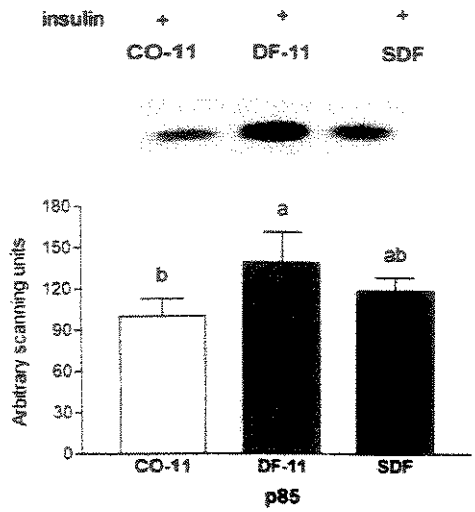

FIGURE 4 Fluorograph obtained after SDS-PAGE of immunoprecipitates of liver from control (CO-11) (n = 8), Mg-deficient (DF-11) (n = 4) and Mg-supplemented (SDF) (n = 6) rats. The rats were injected with saline (not shown) or insulin; 30 s later, liver was excised and homogenized in ice-cold extraction buffer B as described in the Materials and Methods. After centrifugation, aliquots of the supernatant containing equal amounts of protein were immunoprecipitated using anti-insulin receptor substrate (IRS)-1 antibodies and then resolved by SDS-PAGE on 6% polyacrylamide gels. The nitrocellulose transfers were blotted using anti-p85 (phosphatidylinositol 3-kinase) antibody in conjunction with [^{125}I] protein A and then subjected to autoradiography. The means ± SD (n = 4–8) of arbitrary scanning units are depicted at the bottom of the figure. Different letters indicate significant differences, $P < 0.05$.

ylation and in the association/activation between IRS-1 and PI 3-kinase, despite the decreased responsiveness to insulin (Saad 1994). However, when the data were normalized for insulin binding in liver and muscle, reduced receptor phosphorylation (per receptor) was observed in both animal models (Saad et al. 1992). These findings suggest that in insulinopenic diabetes, hypoinsulinemia-induced insulin receptor up-regulation is associated with desensitization of the receptor kinase as a result of chronic hyperglycemia (Sbraccia et al. 1994). In contrast, when hyperglycemia is associated with hyperinsulinemia, the receptor kinase defect is exacerbated, and the insulin resistance is more severe (Mayor et al. 1992). This alteration in insulin receptor tyrosine kinase activity has been attributed to protein kinase C activation in response to elevated intracellular glucose (Draznin et al. 1988, Koya and King 1998). Thus, it is possible that reduced glycemia and insulinemia (Gueux and Rayssiguier 1983) and reduced total area under the glucose and insulin curves (Reis et al., unpublished data) in Mg-deficient rats could act synergistically to hypersensitize the tyrosine kinase of the insulin receptor.

In addition to increased IR tyrosine phosphorylation, an increased number of insulin receptors were observed in DF-11 rats compared with the other groups. This may be explained by an increase in insulin receptor gene transcription in the presence of long-term, reduced insulinemia (Tozzo and Desbuquois 1992), or by alterations in receptor internalization and intracellular degradation (Carpentier 1992).

Although the importance of certain intracellular ions in the control of several cell functions is well established, the role of Mg is not completely known. Many components of the protein synthetic machinery are sensitive to changes in Mg concentrations (Barnes et al. 1995, Zieve et al. 1977). However, the presence of Mg per se is not an absolute requirement for protein synthesis or function in all cell types (Barnes et al. 1995).

As with IR, increased IRS-1 protein and tyrosine phosphorylation levels were observed in the liver of DF-11 rats after insulin stimulation. On the basis of the concept of IRS-1 as the core molecule of a multisubunit signaling complex, high levels of IRS-1 expression could potentially dilute out the effector proteins (Yamauchi and Pessin 1994) and differentially regulate postreceptor processes by preserving the phosphorylation of only some substrates and pathways (Thirone et al. 1998). Studies in Chinese hamster ovary cells overexpressing both IRS-1 and insulin receptors have shown that there may be a delicate balance between the levels of IRS-1 and insulin receptor in the insulin action cascade (Sun et al. 1992). There is evidence that increased expression of the insulin receptor and/or IRS-1 can either enhance or inhibit insulin signaling, depending on the relative levels of IR and IRS-1 and other intracellular components (Yamauchi and Pessin 1994).

As expected, the increase in IRS-1 phosphorylation observed in the liver of DF-11 rats was accompanied by an increase in the association of IRS-1 with the p85 subunit of PI 3-kinase. Although PI 3-kinase activity was not determined, studies on the regulation of enzymatic activity have reported a close correlation with IRS-1 phosphorylation. PI 3-kinase, the best-studied signaling molecule activated by IRS-1, plays an important role in many insulin-regulated metabolic processes, including glucose transport by translocating the intracellular glucose transporter (GLUT 4) to the cell surface (Cheatam et al. 1994, Tsakiridis et al. 1995). Evidence from different sources has demonstrated a correlation between PI 3-kinase activity and glycogen metabolism (Welsh et al. 1994). Thus, the IRS-1/PI 3-kinase pathway may be linked to the activation of glycogen synthesis in liver, and an increase in this association in DF-11 rats may have a role in the enhanced insulin sensitivity of these animals. Knocking out the IR in mouse liver leads to a more pronounced effect on insulin action (i.e., severe insulin resistance) than does knocking out this receptor in muscle (Bruning et al. 1998, Michael et al. 1999). Thus, at least in rodents, the liver plays a more important role in glucose clearance than muscle, what may explain the present data.

Mg supplementation in the SDF group maintained the insulin sensitivity in a manner similar to that of CO-11 rats, and reduced it in comparison to the DF-11 group. Because Mg supplementation in the SCO group did not interfere with the insulin sensitivity as determined by the ivITT, the early steps of insulin action were not studied in this group.

To investigate the mechanism behind the phosphorylation-enhancing action of Mg deficiency, we examined the effect of Mg supplementation in regulating the phosphorylation of these proteins. SDF rats showed values between those of the CO-11 and DF-11 groups for the proteins involved in the early steps of insulin action, with no significant alteration in the sensitivity to insulin in either of the tissues studied.

Thus, the moderate Mg deficiency imposed for 6 wk did not change the sensitivity to insulin relative to control rats as determined by the K_{itt} and Western blot analysis of IR, IRS-1 and the IRS-1/PI 3-kinase association in muscle and liver. In contrast, an enhanced K_{itt} and hepatic insulin sensitivity were observed in rats fed a Mg-deficient diet for 11 wk. This group showed increased insulin-induced protein and tyrosine phosphorylation levels of the IR and IRS-1, as well as increased

IRS-1/PI 3-kinase association compared with the CO-11 group. The maintenance of insulin sensitivity in SDF rats suggested that Mg supplementation in Mg-deficient rats avoided the increase in insulin sensitivity seen in the DF-11 rats. Thus, modulation of the early steps of insulin action in the liver of rats fed a Mg-deficient diet for 11 wk may play a role in the improved sensitivity to insulin seen in this animal model.

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CONCLUSÕES GERAIS

CONCLUSÕES GERAIS

Os resultados obtidos indicam que, nos ratos Wistar:

1. Seis semanas de deficiência de magnésio não alteraram a tolerância à glicose nem a sensibilidade à insulina exógena.
2. Onze semanas de deficiência de magnésio alteraram, significativamente, a tolerância à glicose e a sensibilidade à insulina exógena.
3. A suplementação com magnésio para ratos deficientes evitou o aumento tolerância à glicose e da sensibilidade à insulina.
4. Seis semanas de deficiência de magnésio não alteraram os passos iniciais da sinalização da insulina no fígado e músculo, conforme verificado através da não modificação no nível protéico e grau de fosforilação do receptor de insulina (IR) e do substrato-1 do receptor de insulina (IRS-1) assim como, no grau de associação entre o IRS-1 e a enzima fosfatidilinositol 3-quinase (PI 3-quinase).
5. Onze semanas de deficiência de magnésio alteraram os passos iniciais da sinalização da insulina no fígado, mas não no músculo, conforme avaliado através do aumento no nível protéico e grau de fosforilação do receptor de insulina (IR) e do substrato-1 do receptor de insulina (IRS-1) e, no aumento do grau de associação entre o IRS-1 e a enzima fosfatidilinositol 3-quinase (PI 3-quinase).
6. A suplementação de magnésio para ratos deficientes evitou a alteração dos passos iniciais da ação da insulina no fígado e músculo, como verificado através da não modificação no nível protéico e grau de fosforilação do receptor de insulina (IR) e do substrato-1 do receptor de insulina (IRS-1) e, no aumento do grau de associação entre o IRS-1 e a enzima fosfatidilinositol 3-quinase (PI 3-quinase).
7. A modulação dos passos iniciais da ação da insulina no fígado, induzida por diferentes níveis séricos de magnésio, pode desempenhar um papel importante na homeostase da glicose.

ANEXO 1

ETAPAS INICIAIS DA SINALIZAÇÃO DE INSULINA EM RATOS DEFICIENTES E SUPLEMENTADOS COM MAGNÉSIO

RESUMO APRESENTADO:

XXII CONGRESSO BRASILEIRO DE ENDOCRINOLOGIA E METABOLOGIA

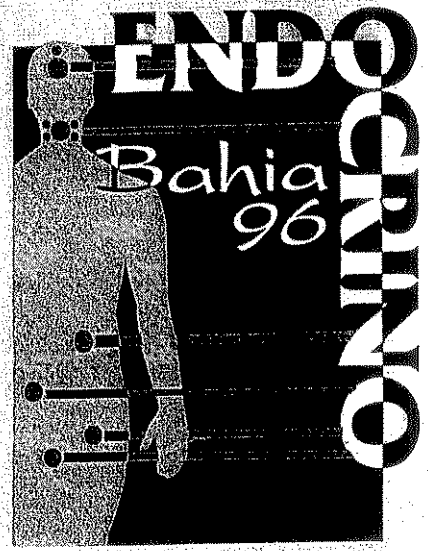
17-21/11/1996– SALVADOR, BA

213

ETAPAS INICIAIS DA SINALIZAÇÃO DA INSULINA EM RATOS DEFICIENTES OU SUPLEMENTADOS COM MAGNÉSIO. Reis, MAB; Reyes, FGR; Latorraca, MQ; Luciano, E; Mota, V; Saad, MJA; Velloso,LA.Deptos.Ed.Física-IB/UNESP/Rio Claro;Nutrição Dietética-FEN/UFMT; Fisiologia e Biofísica-IB,Ciência dos Alimentos-FEA,Clínica Médica-FCM/UNICAMP,Campinas, SP.

A deficiência de magnésio tem sido implicada como fator associado à resistência periférica à insulina, observada em pacientes diabéticos e modelos animais da doença.Neste estudo foram caracterizados os mecanismos moleculares da sinalização da insulina após a depleção e suplementação com Mg.Foram utilizados ratos Wistar machos com 4 semanas de vida, alimentados com dieta Controle (C), contendo ≅507mg Mg/Kg, ou dieta Deficiente em Mg(D), contendo ≅70mg Mg/Kg.Após 6 semanas (na 10a.semana de vida), uma parte dos ratos do grupo D foi transferida para dieta Suplementação (S), contendo ≅2100mg Mg/Kg e mantidos assim por 5 semanas.O restante dos animais foi mantido nas respectivas dietas até o final do experimento.Os níveis de Mg sérico foram determinados por espectrometria com emissão de plasma.A avaliação da fosforilação do receptor de insulina (IR) e de seu principal substrato (IRS-1), assim como da associação IRS-1/PI3q (fosfatidilinositol 3-quinase), em resposta à insulina, nos tecidos hepático e muscular, foi realizada através de imunoprecipitação com anticorpos específicos e imunoblotting com anticorpos antifosfotirosina e anti-PI3q.Os animais do grupo D (de 10sem.de idade) não apresentaram diferença significativa em relação aos do grupo C no ivITT, na fosforilação do IR, IRS-1 e associação IRS-1/PI3q.Nos animais do grupo S (15sem.de idade) observamos aumento para 123,3±33% e 129±32,8% ($p<0,05$) em relação ao grupo C, na fosforilação do IRS-1 no músculo (n=13) e fígado (n=9), respectivamente.A associação IRS-1/PI3q, nesses tecidos, também esteve aumentada no grupo S.Concluímos que a deficiência de Mg imposta aos animais do grupo D não alterou sua sensibilidade à insulina, e quando esse grupo foi suplementado com Mg, a fosforilação do IRS-1 e sua associação com PI3q foram aumentadas.

Certificado



Certificamos que REIS MAB., REYES FGR., LATORRACA MQ., LUCIANO E., MOTA V.V., SAAD MJA.

participou do 22º CONGRESSO BRASILEIRO DE ENDOCRINOLOGIA E METABOLOGIA realizado em Salvador - Bahia, no período de 17 a 21 de novembro de 1996, na qualidade de AUTOR E CO-AUTORES

" ETAPAS INICIAIS DA SINALIZAÇÃO DA INSULINA EM RATOS DEFICIENTES OU SUPLEMENTADOS COM MAGNÉSIO "

Salvador, 21 de novembro de 1996

Thomaz Cruz
Presidente do Congresso

Judith Pousada
Presidente da Comissão Científica

APOIO:

Lilly Diabetes Care

boehringer mannheim

ANEXO 2

MAGNESIUM DEFICIENCY AND SUPPLEMENTATION ON THE EARLY STEPS OF INSULIN SIGNALING

RESUMO APRESENTADO:

XVI INTERNATIONAL CONGRESS OF NUTRITION

27/07 – 01/08/1997 – MONTREAL, CANADA

PR213

MAGNESIUM DEFICIENCY AND SUPPLEMENTATION ON THE EARLY STEPS OF INSULIN SIGNALING.

M.A.B.Reis,F.G.R.Reyes, M.J.A.Saad and L.A.Velloso.IB/FCM/FEA-UNICAMP Campinas-SP, BRAZIL.

Data available suggest that magnesium deficiency may play a role in insulin resistance.In this study we examined the early intracellular events engaged in insulin signaling in magnesium deficient and supplemented rats. Male Wistar rats (4 wk old) were fed either a control diet (C) containing 507mg Mg/Kg or Mg deficient diet (D) containing 70mg Mg/Kg.After 6 wk treatment rats from the D group were changed to a Mg supplemented diet (S) containing 2100mg Mg/Kg.The total period of the study was 11 wk with an interim sacrifice at 6 wk.The serum Mg was measured by inductively coupled plasma emission spectroscopy.The levels and phosphorylation status of insulin receptor (IR) and its substrate IRS-1, as well as the association between this substrate and PI3-kinase, in the liver and muscle, were determined by immunoprecipitation and immunoblotting with anti-peptide antibody to IR, IRS-1, to PI3-kinase and anti-phosphotyrosine antibodies.

At 6 wk, the levels and phosphorylation of IR and IRS-1 and the insulin stimulated IRS-1/PI3-kinase association were similar in the D and C groups.At 11 wk the glucose disappearance rate (Kitt) was reduced only in the S group ($p<0.05$).Insulin stimulation induced increases in IRS-1 phosphorylation and IRS-1/PI3-kinase association in liver and muscle of rats from the S group compared to rats from the C group ($p<0.05$).

Thus, Mg deficiency did not interfere with insulin sensitivity.However, Mg supplementation led to a decay in insulin sensitivity which was accompanied by increases in insulin-induced IRS-1 phosphorylation and IRS-1/PI3-kinase association.

NUTRITION MONTRÉAL 97
IUNS

16th International Congress of Nutrition
16e Congrès international de nutrition

July/juillet 27 - August/août 1, 1997, Montréal, Canada

Marise Reis

PR 213

has participated as a presenter in the poster session of the scientific program of the 16th International Congress of Nutrition which was held in Montréal, Canada, July 27 - August 1, 1997.

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ANEXO 3

EFEITO DA DEFICIÊNCIA CRÔNICA DE Mg SOBRE A SECREÇÃO E AÇÃO DA INSULINA EM RATOS

RESUMO APRESENTADO:

XII REUNIÃO ANUAL DA FEDERAÇÃO DE SOCIEDADES DE BIOLOGIA EXPERIMENTAL - FeSBE

27-30/08/1997 – CAXAMBÚ, MG

08.025

EFEITO DA DEFICIÊNCIA CRÔNICA DE Mg SOBRE A SECREÇÃO E AÇÃO DA INSULINA. [1]Reis,MAB**, [2]Reyes, FG., [3]Saad, MAJ., [3]Velloso, LA. Deptos.Fisiologia/IB, Ciência de Alimentos/FEA, Clínica Médica/FCM/UNICAMP/SP.

Objetivo: Estudos de curta duração, induzindo a deficiência de Mg em animais experimentais e humanos, descrevem alterações na secreção e/ou ação da insulina. Porém, o efeito da deficiência crônica de Mg é pouco claro. Neste trabalho avaliamos a secreção e sensibilidade periférica à insulina em ratos tratados com dieta pobre em Mg por 6 semanas.

Métodos e Resultados: Ratos Wistar machos de 4 sem. de vida foram divididos nos grupos Controle (C) e Deficiente (D) e receberam, respectivamente, dietas contendo 507 mg Mg/Kg e 70 mg Mg/Kg, por 6 sem. A concentração sérica de Mg no jejum foi inferior ($p < 0.05$) no grupo D (2,15 mg/dl ± 0,51 n=26) em relação ao grupo C (2,92 mg/dl ± 0,44 n=19). A área total de glicose (ΔG) em resposta à carga iv de glicose não foi diferente entre os grupos (C= 7391,3 mg/dl.30 min ± 1004,6 n= 8, D= 6935,4 mg/dl. 30 min ± 1092 n= 10). O mesmo aconteceu na resposta secretória de insulina, onde a área total de insulina (ΔI) do grupo C= 50 ng/ml. 30 min ± 14,5 n=8 e D= 62,8 ng/ml. 30 min. ± 23,8 n= 10. Quanto 'a velocidade de decaimento da glicose (Kitt), avaliada nos testes de tolerância iv à insulina e glicose, foi semelhante entre os grupos.

Conclusão: A deficiência de Mg, imposta por 6 sem., não alterou a secreção e ação da insulina, sendo mantida a homeostase glicêmica desses animais.

Apoio Financeiro: CNPq

FeSBE 97

27 a 30 de agosto / Caxambu - MG

Certificamos que

o resumo 08.025 Efeito da deficiência crônica de Mg sobre a secreção e ação da insulina de autoria Reis, M.A.B.; Reyes, F.G.; Saad, M.J.A.; Velloso, L.A foi apresentado na

XII Reunião Anual da Federação de Sociedades de Biologia Experimental-FeSBE, realizada no Hotel Glória na cidade de Caxambu-MG, de 27 a 30 de agosto de 1997.

Comissão Organizadora

ANEXO 4

PROCEDIMENTOS PARA DESCONTAMINAÇÃO DOS MATERIAIS UTILIZADOS NO ENSAIO BIOLÓGICO

PROCEDIMENTOS PARA DESCONTAMINAÇÃO DOS MATERIAIS UTILIZADOS NO ENSAIO BIOLÓGICO

MATERIAIS DE VIDRO, PLÁSTICO OU PORCELANA

Após lavagem habitual com água corrente e detergente Dextran, esses materiais devem permanecer em banho de HNO_3 a 30% ou 10% por 24 h, ou lavagem repetida por 3 vezes. Enxaguar, abundantemente, com água deionizada por 3 vezes.

Obs 1. Recomenda-se banho de HNO_3 a 10% para utensílios que entrarem diretamente em contato com os animais, (por ex. bebedouros ou cochos de vidro), e o banho de HNO_3 a 30% para descontaminação de vidrarias utilizadas nos procedimentos de coleta e armazenagem de material biológico para análise, por ex. tubo de ensaio.
Obs. 2. Verificar se o material plástico é resistente ao banho de HNO_3.

MATERIAIS DE AÇO INÓX (cocho, ponteiras para bebedouro, gaiola metabólica)

Após lavagem habitual com água corrente e detergente Dextran, utensílios desse material devem permanecer em banho com EDTA a 1%, durante 24 h. Após, enxaguar, abundantemente, com água deionizada por 3 vezes.