UNIVERSIDADE
ESTADUAL DE LONDRINA

ALINE FRANCO DA ROCHA

EFEITO DO FATOR DE NECROSE TUMORAL α (TNFα) NA NEOGLICOGÊNESE E NA RESPOSTA HEPÁTICA A INSULINA

Londrina
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Dissertação submetida ao Mestrado em Patologia Experimental da Universidade Estadual de Londrina como requisito parcial à obtenção do título de Mestre.

Orientadora: Prof^a Dr^a Helenir Medri de Souza

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2012

Catalogação elaborada pela Divisão de Processos Técnicos da Biblioteca Central da Universidade Estadual de Londrina

Dados Internacionais de Catalogação-na-Publicação (CIP)

R672e Rocha, Aline Franco.

Efeito do fator de necrose tumoral α (TNFα) na neoglicogênese e na resposta hepática a insulina./ Aline Franco da Rocha. – Londrina, 2012
65 f. : il.

Orientador: Helenir Medri de Souza
Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2010.
Inclui bibliografia.

1. TNFα – Teses. 2. Neoglicogênese – Teses. 3. Insulina. – Teses. 4. Produção hepática de glicose. – Glicogenólise – Teses. 5. Perfusão de fígado. – Teses. I. Souza, Helenir Medri. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. III. Universidade Estadual de Londrina. IV. Título.

CDU 616.092

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Londrina, 4 de maio de 2012.

DEDICATÓRIA

À Deus, acima de tudo, pela graça dispensada
em todos os momentos necessários.

Aos meus pais, Paulo e Eunice,
pelo amor e apoio incondicional aos meus estudos.

AGRADECIMENTOS

Aos meus pais, Paulo Sérgio Pereira e Eunice Lourenço de Oliveira, que sempre acreditaram em mim e apoiaram meus estudos com muito carinho e compreensão.

Aos meus familiares de modo geral, meu irmão Paulo Sérgio Pereira Júnior (Paulinho), Tia Maria Lourenço Ribeiro (Tia Maria), Vô Maria Pereira da Silva (Vô Helena), por sempre estarem ao meu lado orando por mim, me amando e apoiando em todos em momentos.

À Profª Drª Helenir Medri de Souza, primeiramente por ter aceitado ser minha orientadora mesmo sabendo que não era da área e que necessitaria aprender tudo desde o príncipio, pelo imenso tempo e paciência dispensada a me ensinar, por todos os conselhos e orientações que me fizeram chegar até aqui.

À Profª Drª Gláucia Regina Borba Murad que se fez sempre presente com sua sabedoria, seu jeito solícito e sua alegria nestes dois anos.

Aos docentes do Programa de Pós-Graduação em Patologia Experimental, pela cumprida tarefa de ensinar.

Às colegas de laboratório, Daniele Romani Miksza, Hely de Morais, Camila de Souza Oliveira, Mirian Ayumi Kurauti, Flaviane Silva, Thaís Fernanda Liboni, primeiramente pela ajuda sempre presente em dias de experimentos, mas principalmente pela oportunidade de ter feito amigas tão especiais.

À Thaís Fernanda Liboni pela parceria nos experimentos e por todos os momentos, tantos os difíceis como os alegres, que dividimos durante estes dois anos.

À minha turma de mestrado pelo aprendizado em conjunto e pelos ótimos momentos compartilhados.

À Leopoldina Marques (Dina) por sua hospitalidade e amizade.

Ao meu namorado, Sidnei Leles Cardozo, pela compreensão e apoio.

À CAPES, pela concessão da bolsa, possibilitando minha permanência no mestrado.

Filho meu, se aceitares as minhas palavras
e esconderes contigo os meus
mandamentos,
para fazeres atento à sabedoria o teu ouvido
e para inclinares o coração ao
entendimento,
e se clamares por inteligência,
e por entendimento alçares a voz,
se buscares a sabedoria como prata
e como tesouros escondidos a procurares,
então entenderás o temor do Senhor
e acharás o conhecimento de Deus.
Porque o Senhor dá a sabedoria, e da sua
boca vem a inteligência e o entendimento.
Pv 2: 1-6

Rocha, Aline Franco. **Efeito do fator de necrose tumoral α (TNFα) na neoglicogênese e na resposta hepática a insulina.** 2012. 65f. Dissertação (Mestrado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2012.

RESUMO

O fator de necrose tumoral α (TNFα) é uma citocina pró-inflamatória multifuncional, envolvida em muitas respostas biológicas tanto no estado normal como em situações patológicas, que exerce efeitos importantes no metabolismo de proteínas, lipídeos e carboidratos. Uma produção aumentada de TNFα como ocorre no câncer, no diabetes tipo 2 e na obesidade tem sido correlacionada a muitas anormalidades metabólicas presentes nestas doenças. Contudo, uma associação entre o TNFα e as alterações da neoglicogênese hepática em portadores de câncer ainda não foi estabelecida. De fato, o efeito do TNFα na neoglicogênese hepática tem sido pouco investigado e os resultados disponíveis são controversos. Por outro lado, apesar do efeito bem estabelecido do TNFα na indução de resistência insulínica em vários estados patológicos e na inibição da cascata de sinalização da insulina, há poucos estudos avaliando sua influência sobre os efeitos da insulina no fígado. Diante disto, os objetivos deste trabalho foram avaliar o efeito do TNFα na neoglicogênese hepática a partir de vários precursores de glicose, bem como na ação supressiva da insulina sobre a produção hepática de glicose e glicogenólise estimuladas pelo AMPc. Para tanto, o TNFα (10 μg/Kg) foi administrado endovenosamente (veia peniana) em ratos machos Wistar (200-230 g) e, depois de 1 ou 6 horas, a neoglicogênese e a resposta à insulina no catabolismo do glicogênio foram avaliadas em perfusão de fígado *in situ.* Para avaliação da neoglicogênese e de parâmetros metabólicos relacionados, os fígados de ratos com 24 horas de jejum foram submetidos à perfusão com o tampão Krebs-Hanseleit (KH) contendo o precursor neoglicogênico (alanina 2,5 mM; lactato 2 mM; glutamina 5 mM ou glicerol 2 mM). Para avaliação da resposta hepática à insulina no catabolismo do glicogênio estimulado pelo AMPc, os fígados de ratos alimentados foram submetidos à perfusão com KH contendo AMPc (3 μM), na ausência ou presença de concentrações fisiológicas (20 μU/mL) ou suprafisiológicas (500 μU/mL) de insulina. Em todos os experimentos, amostras do perfusado efluente do fígado foram coletadas para determinação da produção hepática de glicose, lactato, piruvato e uréia e da taxa de glicogenólise. Com relação aos efeitos do TNFα na neoglicogênese, a sua administração 6 horas antes da perfusão do fígado, reduziu a produção hepática de glicose a partir da alanina ($p<0,001$) e do lactato ($p<0,01$). O TNFα também aumentou ($p<0,01$) a produção hepática de piruvato, tendeu aumentar a produção de lactato e não teve efeito sobre a produção de uréia a partir da alanina e nem na produção de piruvato a partir do lactato. Além disso, o TNFα não alterou a produção hepática de glicose a partir da glutamina e nem a partir do glicerol. Com relação aos efeitos do TNFα na resposta hepática a insulina, a administração de TNFα, 1 hora antes da perfusão, aboliu completamente ($p<0,05$) o efeito supressivo da insulina 20 μU/mL na produção hepática de glicose e glicogenólise estimuladas pelo AMPc. Além disso, sua administração 6 horas antes da perfusão tendeu a diminuir estes efeitos da insulina. Entretanto, a administração de TNFα, 1 hora antes da perfusão, apenas tendeu a reduzir o efeito inibitório da insulina 500 μU/mL na produção hepática de glicose e glicogenólise estimuladas pelo AMPc e sua administração, 6 horas antes da perfusão, não influenciou estes efeitos da insulina. Pode ser concluído que o TNFα, administrado 6 horas antes da perfusão de fígado, inibiu a neoglicogênese hepática a partir da alanina e do lactato, mas não a partir da glutamina e do glicerol, evidenciando um efeito dependente do precursor. Estes resultados reproduzem a inibição da neoglicogênese que tem sido observada em perfusão de fígado de ratos portadores de tumor Walker-256, sugerindo a participação do TNFα nas alterações da neoglicogênese induzida pelo tumor. Pode ser concluído também que o TNFα diminuiu o efeito supressivo da insulina na produção hepática de glicose e glicogenólise estimuladas pelo AMPc, 1 hora após a sua administração, um efeito que ocorreu para concentração fisiológica, mas não para concentração suprafisiológica de insulina. Estes resultados sugerem que o TNFα pode ser um dos fatores que contribui para o aumento da produção hepática de glicose na obesidade, no diabetes tipo 2 e câncer e indicam que altas concentrações de insulina podem reduzir a inibição do TNFα nos efeitos hepáticos da insulina.

Palavras-chave: TNFα. Neoglicogênese. Insulina. Produção hepática de glicose. Glicogenólise. Perfusão de fígado.

Rocha, Aline Franco. **Effect of tumor necrosis factor α (TNFα) in hepatic gluconeogenesis and insulin response.** 2012. 65p. Dissertação (Mestrado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2012.

ABSTRACT

The tumor necrosis factor α (TNFα) is a multifunctional proinflammatory cytokine, involved in many biological responses both in normal and in pathological situations, which has important effects on the metabolism of proteins, lipids and carbohydrates. An increased production of TNFα as occurs in cancer, type 2 diabetes and obesity has been associated with many metabolic abnormalities present in these diseases. However, an association between TNFα and the changes of hepatic gluconeogenesis in cancer has not been established. In fact, the effect of TNFα in hepatic gluconeogenesis has been little investigated and the available results are controversial. Moreover, despite the effect of TNFα in inhibiting the insulin signaling cascade and in induction of insulin resistance in various pathological states to be well established there are few studies evaluating its influence on the effects of insulin in the liver. In view of this, the present study aimed to evaluate the effect of TNFα in hepatic gluconeogenesis from various precursors of glucose, as well as in suppressive action of insulin on hepatic glucose production and glycogenolysis stimulated by cAMP. For this purpose the TNFα (10 mg/kg) was administered intravenously (penile vein) to male Wistar rats (200-230 g) and after 1 or 6 hours, gluconeogenesis and insulin response in glycogen catabolism were evaluated in situ liver perfusion. For evaluation of gluconeogenesis and related metabolic parameters, the livers of rats with 24 hours of fasting were submitted to perfusion with Krebs-Hanseleit (KH) containing the precursor of gluconeogenesis (2.5 mM alanine, 2 mM lactate, 5 mM glutamine, and 2 mM glycerol). To assess the response to insulin in liver glycogen catabolism stimulated by cAMP, livers from fed rats were submitted to perfusion with KH containing cAMP (3 μM) in the absence or presence of physiological concentrations (20 μU/ml) or supraphysiological (500 μU/ml) of insulin. In all experiments, samples of the effluent perfusion fluid were collected for determination the liver production of glucose, lactate, pyruvate and urea and the rate of glycogenolysis. In relation to the effects of TNFα in gluconeogenesis, its administration 6 hours before perfusion of the liver, reduced hepatic glucose production from alanine ($p<0.001$) and lactate ($p<0.01$). The TNFα also increased ($p<0.01$) the hepatic pyruvate production, tended to increase production of lactate and had no effect on the urea production from alanine or in the lactate production from pyruvate. In addition, the TNFα did not affect hepatic glucose production from glutamine, nor from glycerol. In relation to the effects of TNFα in hepatic response to insulin, administration of TNFα, 1 hour before the perfusion completely abolished ($p<0.05$) the supressive effect of insulin 20 μU/mL in hepatic glucose production and glycogenolysis stimulated by cAMP. In addition, their administration 6 hours before perfusion tended to reduce these effects of insulin. However, administration of TNFα, 1 hour before perfusion, only tended to reduce the inhibitory effect of insulin 500 μU/mL in hepatic glucose production and glycogenolysis stimulated by cAMP, and its administration, 6 h before perfusion, not influenced these effects of insulin. It can be concluded that the TNFα administered 6 hours prior to liver perfusion, inhibited hepatic gluconeogenesis from lactate and alanine, but not from the glutamine and glycerol, showing a precursor-dependent effect. These results reproduce the inhibition of gluconeogenesis which has been observed in liver perfusion of Walker-256 tumor-bearing rats, suggesting the involvement of TNFα in changes of the tumor-induced gluconeogenesis. It can be concluded also that the TNFα decreased the suppressive effect of insulin in the hepatic glucose production and glycogenolysis stimulated by cAMP, 1 hour after its administration, an effect that was observed for physiological concentrations, but not for supraphysiological concentration of insulin. These results suggest that TNFα may be one of the factors that contributes to the increased hepatic glucose production in obesity, type 2 diabetes and cancer and indicates that high concentrations of insulin can reduce the inhibition of TNFα in the hepatic effects of insulin.

Keywords: TNFα. Gluconeogenesis. Insulin. Hepatic glucose production. Glycogenolysis. Liver perfusion.

LISTA DE FIGURAS

ARTIGO 1

ARTIGO 2

LISTA DE ABREVIATURAS E SIGLAS

AMPc	Adenosina monofosfato cíclica
ANOVA	Análise de Variância Unidimensional
AUC	Área sob a curva
ATP	Adenosina trifosfato
Akt/PKB	Proteína quinase B
GLUT4	Transportador de glicose-4
HGP	Produção hepática de glicose
IR	Receptor de insulina
IRS1	Substrato do receptor de insulina 1
IRS2	Substrato do receptor de insulina 2
JNK1	C-Jun-N-terminal quinase-1
JNK2	C-Jun-N-terminal quinase-2
KHB	Tampão Krebs Henseleit
PEPCK	Fosfoenolpiruvato carboxiquinase
PI3K	Fosfatidilinisitol 3-kinase
PKA	Proteína quinase dependente de AMPc
PDE3B	Fosfodiesterase 3B
NFκB	Fator nuclear kappa B
TNFα	Fator de necrose tumoral α
TNFR1	Receptor de TNFα tipo1
TNFR2	Receptor de TNFα tipo2
SEM	Erro padrão da média
UCP2	Proteína desacopladora 2
UCP3	Proteína desacopladora 3

SUMÁRIO

INTRODUÇÃO

INTRODUÇÃO

⅄ *Fator de necrose tumoral α (TNFα) & Efeitos metabólicos*

O fator de necrose tumoral α (TNFα) é uma citocina pró-inflamatória multifuncional sintetizada principalmente por macrófagos ativados, em resposta a doenças inflamatórias, infecção e injúria, mas produzida também por uma variedade de outros tipos celulares incluindo adipócitos, monócitos, linfócitos, fibroblastos, astrócitos, células musculares, células de Kupffer e células tumorais (Barbara et al., 1996; Baud e Karin, 2001; Szlosarek e Balkwill, 2003; Wu e Zhou, 2010).

O TNFα é sintetizado como uma proteína transmembrana de 26 kDa (Wu e Zhou, 2010) e liberado em sua forma solúvel de 17 kDa, após clivagem pela enzima conversora de TNFα, para formar um trímero bioativo circulante de 51 kDa (Bulló-Bonet et al., 1999; Moller, 2000; Aggarwal, 2003). Apesar das diferenças de tamanho e localização, ambas as formas de TNFα, solúvel (17 kDa) e transmembrana (26 kDa), atuam como mediadoras das respostas biológicas e juntas são responsáveis por ações locais, autócrina ou parácrina (17 kDa e 26 kDa), e sistêmicas (17 kDa) desta citocina (Sethi e Hotamisligil, 1999). Suas atividades são mediadas por dois tipos de receptores: o receptor de TNFα tipo 1 (TNFR1), expresso na superfície da maioria dos tipos celulares, e o receptor de TNFα tipo 2 (TNFR2), cuja expressão limita-se às células endoteliais, hematopoéticas e células do sistema imunológico (Aggarwal, 2003; Szlosarek et al, 2003).

Embora a via de transdução de sinal do TNFα seja complexa e não totalmente compreendida, seus efeitos ocorrem principalmente devido a sua capacidade de ativar o fator de transcrição NFκB (fator nuclear kappa B) que leva à transcrição de uma variedade de genes relacionados à inflamação (Bradley, 2008; Wu e Zhou, 2010), os quais promovem, entre outros efeitos, o desenvolvimento de resistência a insulina (Shoelson et al., 2006). Outra via ativada pelo TNFα é a C-Jun N-terminal quinase (JNK). JNK1 e JNK2 podem mediar a fosforilação da serina do substrato do receptor de insulina 1 (IRS1) e desse modo causar resistência a insulina (Fernández-Veledo et al., 2009).

Entre os múltiplos efeitos do TNFα encontram-se a estimulação da produção de outras citocinas, a modulação da função imunológica, a indução de apoptose, a modulação da proliferação e diferenciação celular, bem como uma variedade de efeitos metabólicos (Costelli et al., 1993; Prins et al., 1997; Bulló-Bonet et al., 1999; Warne, 2003; Xanthouela et al., 2004; Schwabe e Brenner, 2006; Kruglov et al., 2008; Tisdale, 2009).

Com relação aos efeitos metabólicos, o TNFα desempenha papel central no metabolismo de proteínas e lipídeos e está também envolvido, entre outros, na neoglicogênese hepática e nas ações da insulina.

Os efeitos do TNFα no metabolismo de proteínas e lipídeos estão bem estabelecidos e, de modo geral, esta citocina estimula o catabolismo e inibe o anabolismo de proteínas e lipídeos. O TNFα é apontado como estimulador da degradação protéica muscular (Carbó et al., 2002) e da lipólise (Zhang et al., 2002; Rydén et al., 2004) e como inibidor da síntese de proteínas (Argilés et al., 2006) e da lipogênese (Warne, 2003). De fato, o TNFα aumentou a concentração de ubiquitina no músculo esquelético (Argilés et al., 2003; Saini et al., 2006), uma molécula que se liga a proteínas direcionando-as para a degradação no sistema ubiquitina-proteassoma (Hasselgren et al., 2002). O TNFα também ativou a lipase hormônio sensível (Arner et al., 1995) e inibiu a lipoproteína lipase endotelial (Berg et al., 1994), efeitos que respectivamente estimulam a lipólise e inibem a lipogênese. Em concordância com estes efeitos, a infusão intravenosa de TNFα promoveu elevação dos ácidos graxos livres e triglicerídeos no plasma (Tredget et al., 1988).

Com relação aos efeitos do TNFα na neoglicogênese hepática, poucos estudos têm sido realizados e os resultados disponíveis são controversos. Há relatos que a administração de TNFα inibiu a neoglicogênese a partir da alanina em perfusão de fígado (Kelmer-Bracht et al., 2006) e que o TNFα inibiu a neoglicogênese a partir do lactato em cultura de hepátocitos (Caton et al., 2009). O TNFα também inibiu a neoglicogênese, estimulada pelo glucagon, a partir do lactato em cultura de hepátocitos (Christ e Nath, 1996). Em concordância, o TNFα reduziu a expressão ou atividade de enzimas chaves da neoglicogênese, como a fosfoenolpiruvato carboxiquinase (PEPCK) (Hill e McCallum, 1992; Battelino et al., 1999; Goto et al., 2001) e a glicose 6-fosfatase (Yasmineh e Theologides, 1992; Metzger et al.,1997; Grempler et al., 2004). Porém, diferente dos achados anteriores, a administração de TNFα aumentou a neoglicogênese hepática a partir da alanina em hepatócitos isolados (Blumberg al., 1995), o que evidencia a falta de consenso dos efeitos do TNFα na neoglicogênese hepática.

No que se refere aos efeitos do TNFα nas ações da insulina, esta citocina tem sido apontada como desencadeadora de resistência insulínica. A administração de TNFα induziu resistência a insulina (Lang et al, 1992; Hotamisligil et al.,1994; Plomgaard et al., 2005; Argilés et al., 2006) no músculo (Del Aguila et al., 1999; Rask-Madsen, 2003; Plomgaard, 2005) e tecido adiposo (Porter et al., 2002) e a sua neutralização reverteu a resistência insulínica nestes tecidos (Uysal et al., 1998; Borst et al., 2004). O TNFα também reduziu a sensibilidade à insulina em cultura de adipócitos (Engelman et al., 2000).

Vários mecanismos são propostos para explicar como o TNFα leva a resistência insulínica, sendo o principal através do seu efeito direto na cascata de sinalização da insulina. O TNFα promove ativação de serinas quinases, enzimas que fosforilam o receptor de insulina (IR) e o IRS1 em resíduos de serina, interferindo na fosforilação da tirosina do IR e do IRS1, diminuindo assim a transmissão do sinal da insulina (Hotamisligil et al.,1994).

O TNFα parece também diminuir a expressão (“down-regulation”) do receptor de insulina, do IRS1 e do transportador de glicose GLUT4 dos adipócitos e das células musculares (Hotamisligil et al., 1993; Warne, 2003), porém não há consenso quanto a isto.

O TNFα induziu alterações na sinalização insulínica, por ativação de serinas quinases, em vários tipos celulares como em adipócitos (Hotamisligil et al., 1996), células musculares (Del Aguila et al., 1999) e células hepáticas (Gupta et al., 2007). A incubação de hepatócitos com TNFα induziu a fosforilação do IRS1 em resíduos de serina com sua conseqüente degradação (Gupta et al, 2007) e a incubação de células de hepatoma com TNFα diminuiu a fosforilação da tirosina do IR e do IRS1 (Feinstein et al., 1993). Similarmente, a incubação de adipócitos com TNFα diminuiu a fosforilação do IR e do IRS1 com conseqüente redução na captação de glicose estimulada pela insulina (Hotamisligil et al., 1999). Além disso, a neutralização do TNFα aumentou a autofosforilação do IR e do IRS1 no tecido adiposo e muscular, embora não promoveu nenhuma alteração na sinalização da insulina no fígado (Hotamisligil et al.,1994). Em conjunto, estas evidências demonstram o efeito bem estabelecido do TNFα na indução de resistência a insulina.

⅄ *Fator de necrose tumoral α (TNFα) & Anormalidades metabólicas*

O TNFα tem atraído atenção considerável, não somente por estar envolvido em muitas respostas metabólicas no estado normal, mas também por estar correlacionado a muitas anormalidades metabólicas associadas a várias doenças.

De fato, pacientes em situações patológicas associadas à alta produção de TNFα, como câncer, obesidade e diabetes *mellitus* tipo 2, apresentam anormalidades no metabolismo de lipídeos, proteínas e carboidratos (Hotamisligil, 1999; Moller, 2000; Tisdale, 2009).

Uma produção aumentada de TNFα como ocorre no câncer (Nakashima et al., 1998; Ferrajoli et al., 2002; Berberoglu et al., 2004; Michalaki et al., 2004; Rebeca et al., 2008) está associada a estados catabólicos conduzindo a síndrome da caquexia do câncer (Hotamisligil et al., 1993), caracterizada por depleção de massa muscular esquelética (Argilés et al., 2006) e do tecido adiposo (Warne, 2003) e por acentuada perda de peso corpóreo (Gelin et al., 1991; Matthys e Billiau, 1997; Argilés et al., 2006; Tisdale, 2010). O TNFα está implicado na depleção de massa muscular e adiposa associada ao câncer por estimular a proteólise e a lipólise, por inibir a lipogênese e a adipogênese e também por aumentar a apoptose do músculo esquelético, de adipócitos e pré-adipócitos (Warne, 2003; Argilés et al., 2006). O TNFα, também denominado de caquectina, parece ser o principal mediador da caquexia associada ao câncer.

É possível que o TNFα também esteja associado às alterações da neoglicogênese em portadores de câncer. Neoglicogênese aumentada (Waterhouse et al., 1979; Lundholm et al., 1982; Shearer et al., 1983; Roh et al., 1984; Blumberg et al., 1993; Fischer et al., 1997) ou diminuída (Acco et al., 2007; Corbello-Pereira et al., 2004; Liu et al*.,* 1990) tem sido relatada em portadores de câncer e efeitos inibitórios (Christ e Nath, 1996; Kelmer-Bracht et al., 2006; Caton et al., 2009) e estimulatórios (Blumberg al., 1995) do TNFα na neoglicogênese têm também sido encontrados. Diante dos poucos estudos realizados e dos efeitos aparentemente contraditórios do TNFα na neoglicogênese hepática, uma correlação entre o TNFα e as alterações da neoglicogênese no câncer ainda não foi estabelecida.

Uma produção aumentada de TNFα em portadores câncer e níveis séricos elevados de TNFα em pacientes com diabetes tipo 2 (Mishima et al., 2001) e em modelos experimentais de diabetes tipo 2 e obesidade (Hotamisligil, 1999) parece também estar associados ao desenvolvimento de resistência a insulina nestas situações patológicas.

Pacientes com vários tipos de cânceres apresentam muitas anormalidades metabólicas do diabetes tipo 2 como: resistência a insulina, intolerância à glicose e maior produção e liberação hepática de glicose provenientes da neoglicogênese e glicogenólise (Lundholm et al., 1978; Copeland et al., 1987; Rofe et al., 1994; Tayek et al., 1992). Embora os mecanismos relacionados ao desenvolvimento de resistência a insulina em portadores de câncer ainda não estão bem esclarecidos a resistência insulínica em pacientes com câncer gastrointestinal foi correlacionada com a concentração de TNFα no soro e a administração de TNFα causou resistência a insulina em humanos (Mccall et al., 1992).

Estudos têm demonstrado que o TNFα possui função chave no desenvolvimento de resistência a insulina em portadores de diabetes tipo 2, tanto em hepatócitos (Hotamisligil, 1999; Torisu et al., 2007), como nas células adiposas (Hotamisligil, 1999; Yoshikawa et al., 1999; Patiag et al., 2000; Warne, 2003; Mlinar et al., 2007) e no músculo esquelético (Plomgaard et al., 2005). A infusão de TNFα prejudicou a supressão da produção hepática de glicose mediada pela insulina (Lang et al., 1992) e a neutralização do TNFα em ratos obesos restaurou a sensibilidade insulínica e reverteu completamente a supressão da produção hepática de glicose (Cheung et al., 1998). O bloqueio da expressão gênica do TNFα também melhorou a sensibilidade à insulina em modelo de obesidade (Uysal et al., 1997). A resistência hepática a insulina induzida pelo TNFα resulta em aumento da produção de glicose pelo fígado e hiperglicemia, anormalidades presentes no diabetes tipo 2 (Uysal et al., 1997).

A obesidade é a condição clínica mais comum associada com o diabetes tipo 2 (Permutt et al, 2005; Grundy, 2004) e fatores pró-inflamatórias produzidos pelo tecido adiposo desempenham papel importante na associação entre obesidade e diabetes tipo 2 (Hotamisligil, 2006). Pacientes obesos possuem altos níveis circulantes de TNFα e são propensos a desenvolverem diabetes tipo 2 devido, entre outros fatores, à resistência insulínica mediada pelo TNFα (Hotamisligil et al., 1993). Vários estudos têm mostrado que o TNFα produzido pelo tecido adiposo é capaz de induzir resistência a insulina (efeito autócrino), ativando serinas quinases que dificultam a transdução do sinal da insulina (Hotamisligil et al., 1994; Hotamisligil et al., 1993; Hirosumi et al., 2002; Shoelson et al., 2003). Além disso, tem também sido demonstrado o efeito do TNFα produzido localmente (efeito parácrino), inibindo a ação da insulina (De Souza et al., 2005; Farrell e Larter et al., 2006). Assim, o TNFα tem sido implicado como elo entre obesidade e diabetes tipo 2 (Gupta et al., 2007).

Em síntese, o TNFα exerce uma variedade de efeitos importantes no metabolismo de proteínas, lipídeos e carboidratos, tanto no estado normal como em situações patológicas, alguns muito estudados e bem estabelecidos e outros não.

Considerando que o efeito do TNFα na neoglicogênese hepática tem sido pouco investigado e que os resultados disponíveis são controversos e considerando também que, apesar do efeito bem estabelecido do TNFα na sinalização da insulina, não há estudos avaliando a sua influência no efeito supressivo da insulina sobre o catabolismo do glicogênio hepático estimulado pela adenosina monofosfato cíclica (AMPc), seguem os objetivos do presente trabalho.

OBJETIVOS

OBJETIVOS

Avaliar, em estudos de perfusão de fígado *in situ*, o efeito da administração endovenosa do TNFα sobre:

1. A neoglicogênese hepática a partir de vários precursores de glicose (alanina, lactato, glutamina e glicerol).

*Os resultados destes estudos resultaram na elaboração do primeiro artigo científico, em anexo, a ser submetido para publicação na revista “Pharmacological Reports”.

2. A resposta hepática a insulina, particularmente sobre o efeito supressivo da insulina no catabolismo do glicogênio estimulado pelo AMPc.

*Os resultados destes estudos resultaram na elaboração do segundo artigo científico, em anexo, a ser submetido para publicação na revista “Molecular and Cellular Biochemistry”.

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ARTIGO 1

Inhibitory effect of tumor necrosis factor α on hepatic gluconeogenesis is dependent on the precursor

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Abstract: Tumor necrosis factor α (TNFα) is a cytokine involved in many metabolic responses in both normal and pathological states. Increased production of TNFα in cancer has been correlated to many metabolic abnormalities associated with this disease. Here, we examined the influence of TNFα on hepatic gluconeogenesis from various precursors of glucose. TNFα (10 μg/Kg) was administered intravenously in rats, and after 6 h, gluconeogenesis from alanine, lactate, glutamine and glycerol, and several related metabolic parameters, were evaluated in liver perfusion. Administration of TNFα reduced the hepatic glucose production from alanine ($p<0.001$) and from lactate ($p<0.01$). TNFα also increased ($p<0.01$) the liver production of pyruvate, tended to increase the lactate production and had no effect on the urea production from alanine and on the pyruvate production from lactate. In addition, TNFα did not alter the hepatic glucose production from glutamine nor from glycerol. It can be concluded that TNFα inhibited hepatic gluconeogenesis from alanine and lactate, but not from glutamine and glycerol, evidencing a precursor-dependent effect of TNFα on gluconeogenesis. These results reproduce the inhibition of gluconeogenesis that has been observed in perfused liver of Walker-256 tumor-bearing rats, suggesting the involvement of TNFα on changes of the gluconeogenesis induced by tumor.

Keywords: TNFα. Gluconeogenesis. Alanine. Lactate. Glutamine. Glycerol.

INTRODUCTION

Tumor necrosis factor α (TNFα) is a multifunctional cytokine that exerts a variety of important metabolic effects in both normal and pathological states (Pfeffer, 2003; Kruglov et al., 2008; Bulló-Bonet et al., 1999).

TNFα stimulates the muscle protein degradation (Carbó et al., 2002) and lipolysis (Zhang et al. 2002; Rydén et al., 2004; Chen et al., 2009) and inhibits the protein synthesis (Argilés et al., 2006) and lipogenesis (Warne, 2003). TNFα is also associated with increased apoptosis of skeletal muscle cells (Tisdale, 2009;

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Costelli et al., 1993), adipocytes and preadipocytes (Warne, 2003; Prins et al, 1997). Thus, an increased production of TNFα, as it occurs in cancer (Rebeca et al., 2008; Ferrajoli et al., 2002; Michalaki et al., 2004; Berberoglu et al., 2004; Nakashima et al., 1998) causes depletion of skeletal muscle mass (Argilés et al., 2005) and adipose tissue (Warne, 2003) and severe loss of body mass (cancer cachexia) (Tisdale, 2010; Argilés et al., 2005; Matthys e Billiau, 1997; Gelin et al., 1991).

In spite of the fact that TNFα has been related to many metabolic abnormalities associated with cancer, the association of TNFα with the changes of gluconeogenesis in cancer, which has been reported to be increased (Waterhouse et al., 1979; Lundholm et al., 1982; Shearer et al., 1983; Roh et al., 1984; Blumberg et al., 1993; Shearer et al., 1983; Fischer et al., 1997) or decreased (Acco et al., 2007; Corbello-Pereira et al., 2004; Liu et al*.,* 1990) in tumor-bearing state, is not established.

In fact, the effect of TNFα on hepatic gluconeogenesis has been little investigated and the available results are inconclusive. There are reports that administration of TNFα inhibited gluconeogenesis from alanine in liver perfusion (Kelmer-Bracht et al., 2006). TNFα also inhibited gluconeogenesis from lactate in cultured hepatocytes (Caton et al., 2009; Christ e Nath., 1996). Accordingly, TNFα reduced the expression or the activity of key enzymes of gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) (Hill e McCallum, 1992; Goto et al., 2001; Battelino et al., 1999) and glucose 6-phosphatase (Grempler et al., 2004; Metzger et al., 1997; Yasmineh e Theologides, 1992). However, administration of TNFα increased gluconeogenesis from alanine in isolated hepatocytes (Blumberg et al., 1995). Since the effect of TNFα on gluconeogenesis is controversial we evaluated the effect of administration of TNF on gluconeogenesis from alanine, lactate, glutamine and glycerol in perfused rat liver.

MATERIALS AND METHODS

Chemicals

Recombinant rat TNFα was purchased from Peprotech (Mexico SA). Aliquots of reconstituted TNFα were stored at -20°C and thawed immediately before the experiments. Gluconeogenic precursors and all other chemicals (98-99.8% purity) were purchased from Sigma Chemical Co. (St Louis, USA), Acros Organics (New Jersey, USA), Reagen (Rio de Janeiro, Brazil) and Merck (Darmstad, Germany).

Animals and administration of TNFα

Male Wistar rats (200-230g), kept at a constant temperature (23±2°C), in a light/dark cycle of 12 h and fed with a standard commercial laboratory diet (Nuvilab$^{®}$), were used in all experiments. Rats were submitted to 24 h of fasting before the liver perfusion to remove the interference of liver glycogen.

TNFα (10 µg/Kg) was administered in rats, 6 h before the liver perfusion. The administration consisted of endovenous injection into the penial vein of 200 µl of a buffered saline solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM, KH_2PO_4 and 0.1% bovine serum albumin), pH 7.4, containing 2 µg TNFα. Control rats were injected with 200 µl buffered saline. All protocols with animals were approved by the Ethics Committee for Animal Experimentation of the State University of Londrina.

Liver perfusion

For the surgical procedure, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) and submitted to *in situ* liver perfusion, as previously described (Mario et. al, 2009; Leonardo et al., 2009). The perfusion fluid, Krebs-Henseleit buffer (KHB), pH 7.4, at 37ºC and saturated with a 95%:5% O_2:CO_2 mixture, was introduced in the liver (4 mL/min per gram of liver) through a cannula inserted into the portal vein, while a second cannula in the inferior vena cava was used to collect the effluent perfusate. The composition of the KHB buffer was: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 2.5 mM $CaCl_2$. Gluconeogenic precursors were dissolved in

KHB. Livers were perfused with KHB during 10 min and with KHB plus alanine (2.5 mM), lactate (2 mM) or glycerol (2 mM) during 30 min or with glutamine (2.5 mM) during 80 min. The effluent perfusate was collected every 2 or 5 min to measure the production of glucose, lactate, pyruvate or urea. At the end of the perfusion, the liver was removed and weighed, to allow precise metabolic calculations and the correction of flux rates. The differences in production of glucose, lactate, pyruvate and urea, before and during infusion of gluconeogenic precursor, were used to calculate the areas under curves (AUCs).

Analytical procedures

The concentrations of glucose (Bergmeyer and Bernt, 1974), pyruvate (Czok and Lamprecht, 1974), lactate (Gutmann and Wahlefeld, 1974) and urea (Gutmann and Bergmeyer, 1974) in the effluent perfusion fluid of the liver were assayed by standard enzymatic methods.

Statistical procedures

Normal distribution and variance homogeneity were tested and Student's *t*-test was employed to analyze the results. Statistical analysis was carried out with the program Statistic 6.0 or GraphPad Prism 4.0, at the 5% level of significance ($p<0.05$). Data are expressed as mean ± standard error of the mean *(*SEM*).*

RESULTS

Infusion of alanine (2.5 mM) in the liver, at 10 minutes of the perfusion period, increased the production of glucose in rats injected with TNFα and controls (Figure 1A). However, this increase was lower ($p<0.001$) in rats injected with TNFα, as demonstrated by AUCs (Figure 1B).

Infusion of alanine in the liver also increased the production of pyruvate (Figure 2A), lactate (Figure 2B) and urea (Figure 2C) in both groups.

However, the production of pyruvate was higher ($p<0.01$) and the production of lactate tended to be higher in rats injected with TNFα compared to the controls, while the production of urea was similar in both groups, as evidenced by the respective AUCs (Figure 2D).

Infusion of lactate (2 mM) in the liver, similar to alanine, promoted lower ($p<0.01$) increase in the production of glucose in rats injected with TNFα than in controls (Figure 3A), as illustrated by AUCs (Figure 3B). However, the production of pyruvate from lactate was similar in both groups (Figures 3C and 3D).

In contrast to alanine and lactate, infusion of glutamine (2.5 mM) and glycerol (2 mM) in the liver promoted similar increase in the production of glucose in both groups (Figures 4A and 5A), as revealed by AUCs (Figures 4B and 5B).

DISCUSSION

Decreased hepatic glucose production from alanine in rats injected with TNFα (Figure 1B) shows the inhibitory effect of this cytokine on gluconeogenesis from this precursor. Moreover, increased production of pyruvate, tendency to increased production of lactate and lack of effect of TNFα on the production of urea (Figure 2D) indicates that this inhibitory effect of TNFα on gluconeogenesis did not involve reduction of hepatic uptake of alanine nor inhibition of alanine aminotransferase. In fact, increased production of pyruvate and tendency to increased production of lactate (Figure 2D) suggest increased hepatic uptake of alanine in rats injected with TNFα. Accordingly, administration of antibody to TNFα attenuated the increased transport of amino acids in tumor-bearing rats (Inoue et al., 1995) and TNFα potentiated the action of glucagon in the hepatic uptake of amino acids (de Bandt et al., 1994). Although increased production of pyruvate and tendency to increased production of lactate (Figure 2D) could indicate that the inhibitory effect of TNFα on gluconeogenesis from alanine might have involved reduction of the rate of transport of pyruvate across the mitochondrial membrane, it probably did not occur, since the production of pyruvate from lactate was not altered in rats injected with TNFα (Figure 3D).

Similarly to alanine, administration of TNFα inhibited gluconeogenesis from lactate (Figure 3B), an effect that was probably not due the lower hepatic uptake of lactate nor the inhibition of lactate dehydrogenase, as indicated by the lack of effect of TNFα on the production of pyruvate (Figure 3D).

Although several studies have demonstrated the inhibitory effect of TNFα on key enzymes of gluconeogenesis, such as PEPCK (Hill e McCallum, 1992; Goto et al., 2001; Battelino et al., 1999; Chist e Nath, 1996; Caton et al., 2009) and glucose 6-phosphatase (Grempler et al., 2004; Metzger et al.,1997; Yasmineh and Theologides, 1992), the lack of effect of TNFα on hepatic gluconeogenesis from glutamine (Figure 4) and glycerol (Figure 5) suggests that PEPCK, fructose 1,6-bisphosphatase and glucose 6-phosphatase were not inhibited by TNFα under the experimental conditions of the present study. Corroborating our results, the activity of PEPCK was unchanged 6 h after injection of TNFα, but it was reduced 12 h after its administration (Metzger et al., 1997), demonstrating a time-dependent effect of TNFα on the activity of PEPCK.

Thus, inhibition of gluconeogenesis from alanine and lactate by TNFα must have been caused by changes in other variables. It is possible that TNFα may have affected mitochondrial steps of the gluconeogenic pathway, such as the conversion of pyruvate to oxaloacetate by pyruvate carboxylase, an ATP-dependent reaction. It was verified in isolated mitochondria that TNFα induced the uncoupling of mitochondrial respiration (Busquets et al., 2003), an effect that reduces the ATP synthesis. Indeed, administration of TNFα in rats caused an increase in the levels of mRNA of uncoupling proteins (UCP2 and UCP3) in skeletal muscle (Busquets et al., 1998).

In agreement with our results, TNFα inhibited gluconeogenesis from alanine in perfused rat liver (Kelmer-Bracht al., 2006) and from lactate in cultured hepatocytes (Caton et al., 2009). TNFα also inhibited gluconeogenesis, stimulated by glucagon, from lactate in cultured hepatocytes (Christ e Nath, 1996). Nevertheless, unlike our results, the administration of TNFα increased gluconeogenesis from alanine in isolated hepatocytes (Blumberg al., 1995). However in these studies, high concentrations of TNFα, 25 times higher than the present study, were used.

These findings suggest that the effect of TNFα on gluconeogenesis, stimulatory or inhibitory, may be dependent of its concentration. In cancer, the concentration of TNFα could range with the disease stage, size and type of tumor. This could explain, at least in part, the lack of consensus related to the hepatic gluconeogenesis in cancer, which has been reported to be increased (Waterhouse et al., 1979; Lundholm et al., 1982; Shearer et al., 1983; Roh et al., 1984; Blumberg et al., 1993; Shearer et al., 1983; Fischer et al., 1997) or decreased (Acco et al., 2007; Corbello-Pereira et al., 2004; Liu et al*.,* 1990).

It can be concluded that TNFα inhibited hepatic gluconeogenesis from alanine and lactate, but not from glutamine and glycerol, demonstrating that the inhibitory effect of TNFα on hepatic gluconeogenesis is dependent on the precursor. These results reproduce those previously found in perfused liver of tumor-bearing rats, namely, inhibition of gluconeogenesis from alanine (Acco et al., 2007; Corbello-Pereira et al., 2004; Liu et al*.,* 1990) and from lactate+pyruvate (Veiga et al., 2007), but not from glutamine (Corbello-Pereira et al., 2004) and glycerol, suggesting the involvement of TNFα on changes of the gluconeogenesis induced by tumor.

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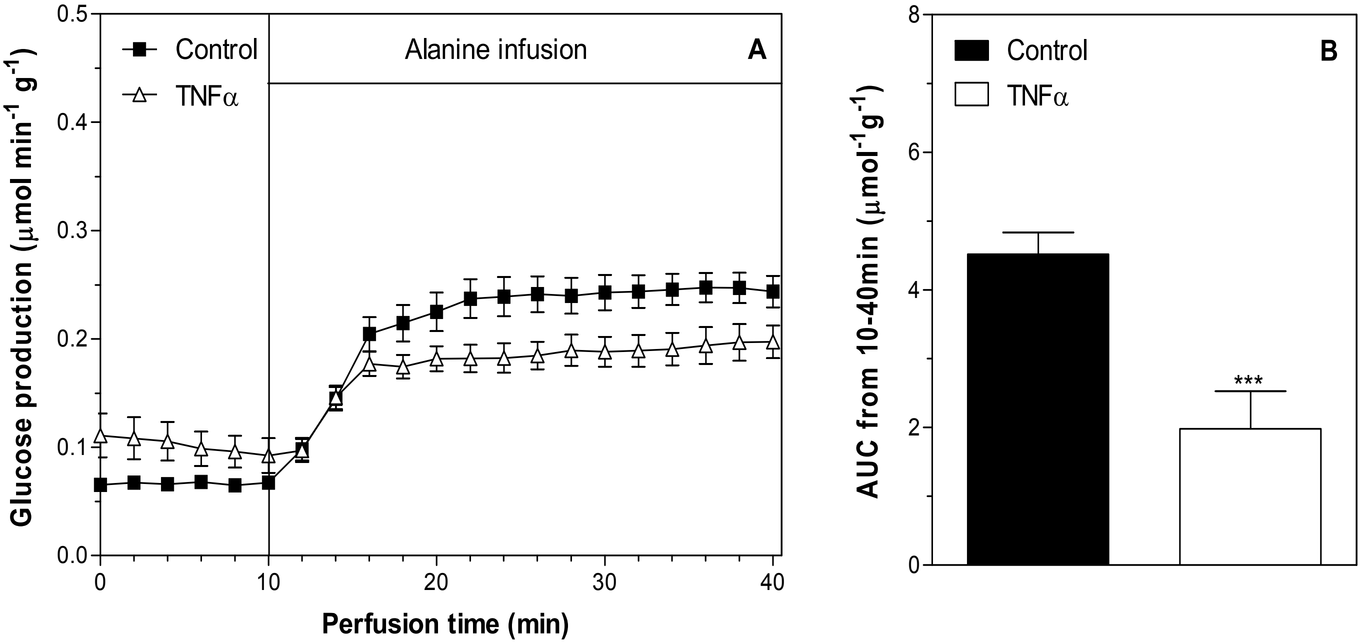

Figure 1 - Effect of tumor necrosis factor α (TNFα) on hepatic production of glucose from alanine (A) and areas under curves (AUCs) (B). Livers were perfused 6 h after injection of TNFα (10 μg/Kg) or buffered saline (Control), as described in Materials and Methods. Alanine (2.5 mM) was infused between 10-40 min. Data are mean ± SEM from 6-13 experiments. ***p<0.001 *vs* control (unpaired Student's test).

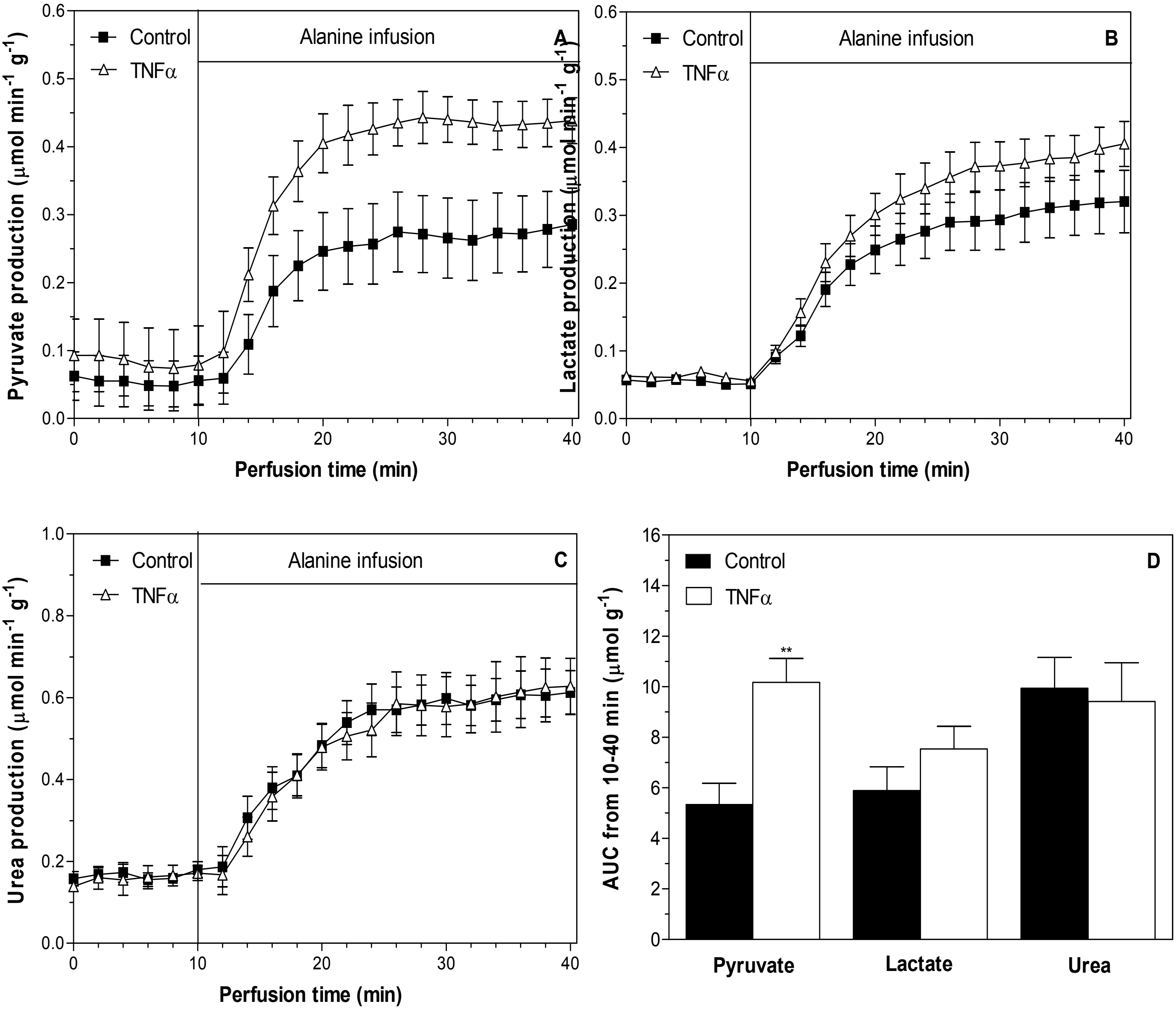

Figure 2 –Effect of tumor necrosis factor α (TNFα) on hepatic production of pyruvate (A), lactate (B) and urea (C) from alanine and the areas under curves (AUCs) (D). Livers were perfused 6 h after injection of TNFα (10 μg/Kg) or buffered saline (Control), as described in Materials and Methods. Alanine (2.5 mM) was infused between 10-40 min. Data are mean ± SEM from 6-13 experiments. **p<0.01 *vs* control (unpaired Student's test).

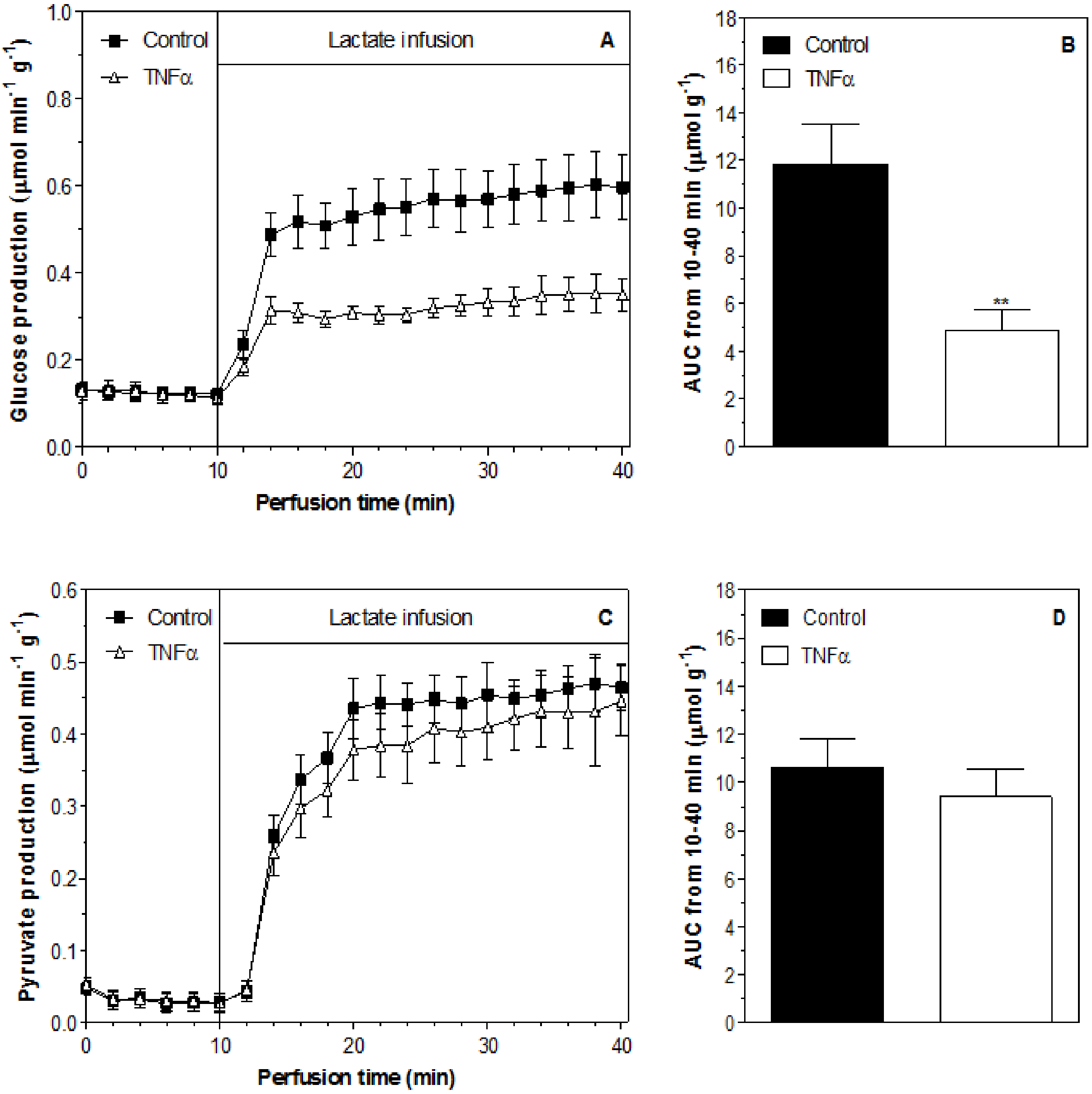

Figure 3 - Effect of tumor necrosis factor α (TNFα) on hepatic production of glucose (A) and pyruvate (C) from lactate and areas under curves (AUCs) (B,D). Livers were perfused 6 h after injection of TNFα (10 µg/Kg) or buffered saline (Control), as described in Materials and Methods. Lactate (2 mM) was infused between 10-40 min. Data are mean ± SEM from 12 experiments. $^{**}p<0.01$ *vs* control (unpaired Student's test).

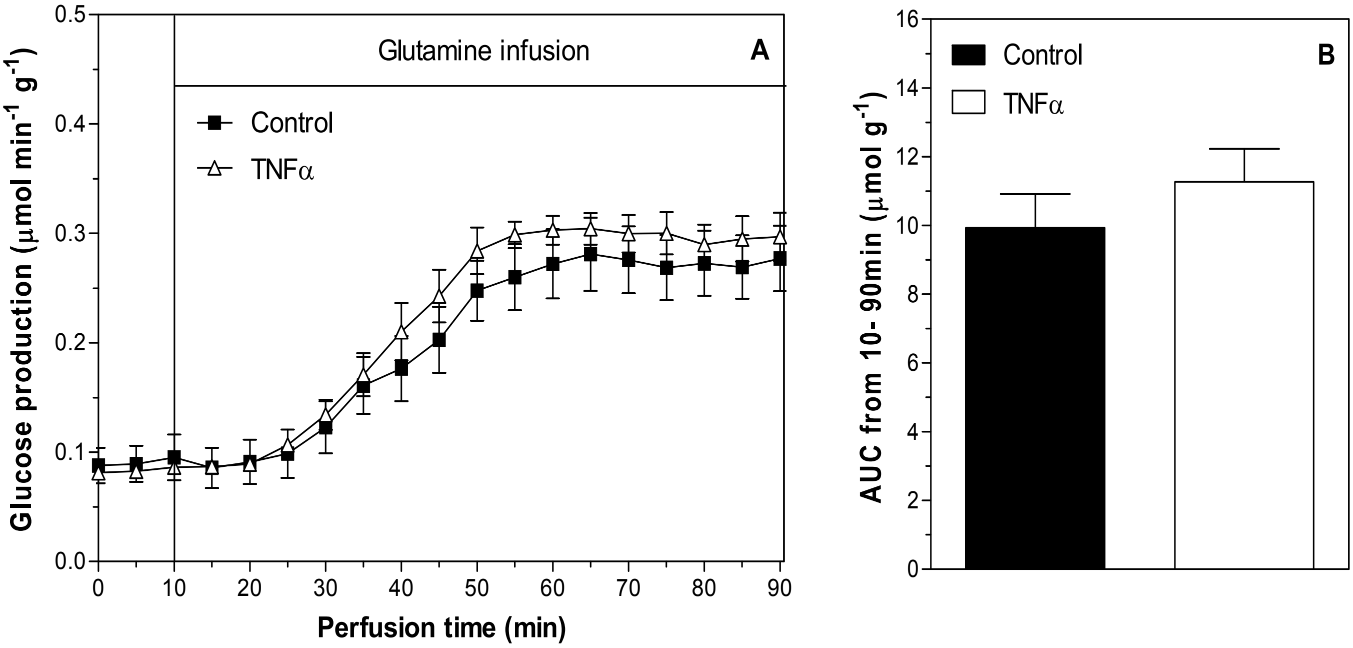

Figure 4 - Effect of tumor necrosis factor α (TNFα) on hepatic production of glucose from glutamine (A) and areas under curves (AUCs) (B). Livers were perfused 6 h after injection of TNFα (10 µg/Kg) or buffered saline (Control), as described in Materials and Methods. Glutamine (2.5 mM) was infused between 10-90 min. Data are mean ± SEM from 5-6 experiments. Diferences between AUCs were analised by the unpaired Student's test.

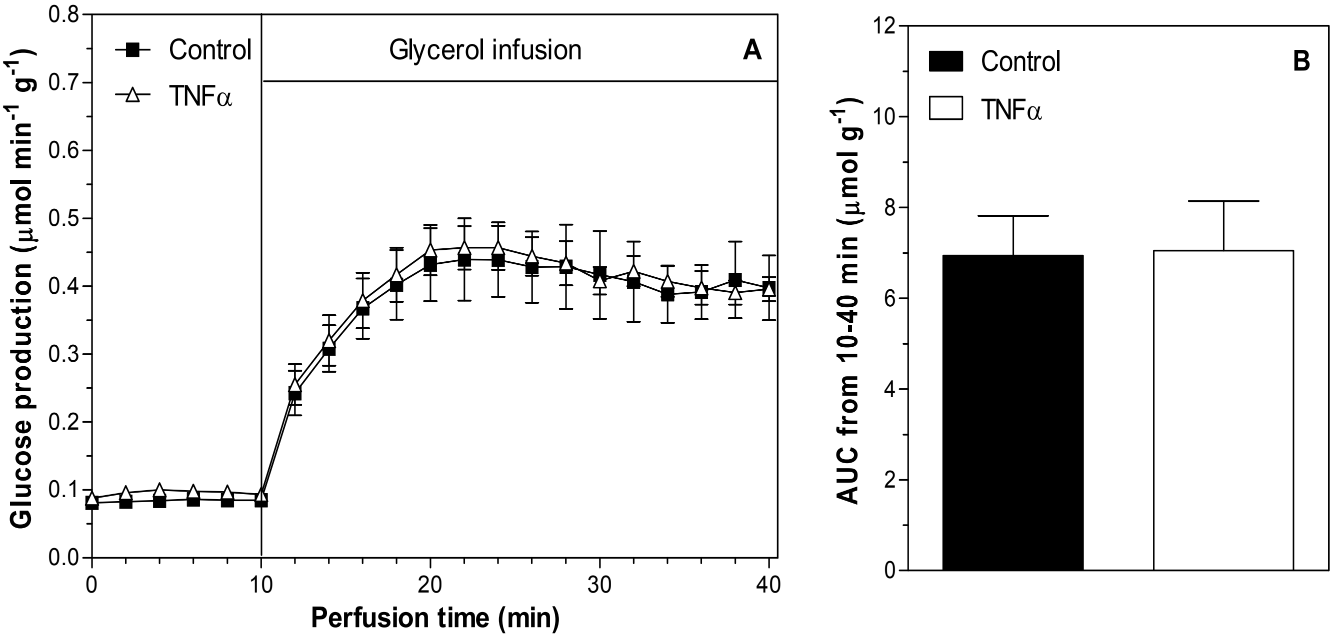

Figure 5 - Effect of tumor necrosis factor α (TNFα) on hepatic production of glucose from glycerol (A) and areas under curves (AUCs) (B). Livers were perfused 6 h after injection of TNFα (10 µg/Kg) or buffered saline (Control), as described in Materials and Methods. Glycerol (2 mM) was infused between 10-40 min. Data are mean ± SEM from 12 experiments. Differences between AUCs were analised by the unpaired Student's test

ARTIGO 2

Tumor necrosis factor α decreased the suppressive effect of insulin on hepatic glucose production and glycogenolysis stimulated by cAMP

Aline Franco da Rocha[1], Helenir Medri de Souza[1*]

Abstract: Tumor necrosis factor α (TNFα) has been implicated in the development of insulin resistance in several pathophysiological states such as diabetes, obesity and cancer. Despite the inhibition of TNFα in insulin signal transduction is well established, there are few informations about its influence on the effects of insulin in the liver. Thus, the aim of this study was to investigate the influence of TNFα on the suppressive effect of insulin on hepatic glucose production (HGP) and glycogenolysis stimulated by cyclic adenosine monophosphate (cAMP). For this purpose, TNFα (10 µg/Kg) was intravenously administered in rats and after 1 or 6 h the livers were submitted to *in situ* perfusion with cAMP (3 µM), in the presence or absence of physiological (20 µU/mL) or supraphysiological (500 µU/mL) concentrations of insulin. Administration of TNFα, 1 h before perfusion, completely abolished ($p<0.05$) the suppressive effect of insulin 20 µU/mL on HGP and glycogenolysis stimulated by cAMP. In addition, administration of TNFα 6 h before perfusion tended to reduce these effects of insulin. However, the administration of TNFα, 1 h before perfusion, only tended to reduce the inhibitory effect of insulin 500 µU/mL on HGP and glycogenolysis stimulated by cAMP and its administration, 6 h before perfusion, did not influence these effects of insulin. In conclusion, TNFα decreased the suppressive effect of insulin on HGP and glycogenolysis stimulated by cAMP, 1 h after its administration, an effect that occurred for physiological concentration, but not for supraphysiological concentration of insulin. These results suggest that TNFα may be one of the factors that contributes to the increase of HGP in obesity, type 2 diabetes and cancer and indicate that high concentrations of insulin can reduce the inhibition of TNFα on hepatic effects of insulin.

Keywords: TNFα. Insulin. Hepatic glucose production. Glycogenolysis. Liver Perfusion.

INTRODUCTION

Tumor necrosis factor α (TNFα) is a multifunctional proinflammatory cytokine, involved in many metabolic responses, that has been implicated as a trigger of insulin resistance. The administration of TNFα induced peripheral insulin

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resistance (Lang et al., 1992), in the adipose tissue (Porter et al., 2002), muscle (Plomgaard, 2005) and hepatocytes (Xu et al., 2008). Accordingly, inhibition of TNFα in obese, by administration of anti-TNFα antibody or by deficiency of its receptor (gene knockout), increased peripheral insulin sensitivity (Hotamisligil et al., 1993; Cheung et al., 1998; Scheyer et al., 1998; Liang et al., 2008), in the muscle (Cheung et al., 1998; Borst et al., 2004) and liver (Cheung et al., 1998; Cheung et al., 2000).

Furthermore, incubation of adipocytes, muscle cells or hepatocytes with TNFα inhibited the insulin signaling cascade (Feinstein et al., 1993; Hotamisligil et al., 1994; Miura et al., 1999; Gupta et al., 2007; Xu et al., 2008). In these studies, TNFα increased serine phosphorylation of insulin receptor (IR) and insulin receptor substrate (IRS-1), probably by activation of serine kinases (Kanety et al., 1995), which reduced tyrosine phosphorylation of IR and IRS-1 stimulated by insulin (Feinstein et al., 1993; Hotamisligil et al., 1994; Miura et al., 1999; Gupta et al., 2007; Xu et al., 2008) and prevented the activation of phosphatidylinositol 3-kinase (PI3K) (Kanety et al., 1995; Del Aguila et al., 1999; Miura et al., 1999) and protein kinase B (Akt/PKB) (Plomgaard, 2005; Xu et al., 2008), essential steps in the insulin signal transduction.

In addition, it has been shown that an increased production of TNFα is implicated in the pathogenesis of insulin resistance in type 2 diabetes and obesity and that an enhanced expression of TNFα by adipose tissue plays an important role in the association between obesity and type 2 diabetes (Hotamisligil et al., 1993; Hotamisligil et al., 1994; Uysal et al., 1997; Moller, 2000).

An increased production of TNFα seems to be also implicated in insulin resistance in cancer (Tayek, 1992; Rofe et al., 1994; Noguchi et al., 1998). Insulin resistance in patients with cancer was associated with increased circulating levels of TNFα (Mccall et al., 1992) or with increased expression of this cytokine in the muscle (Noguchi et al., 1998).

Despite the well established effect of TNFα on the insulin signaling cascade and on the induction of insulin resistance in various pathological states, there are few studies evaluating its influence on the effects of insulin on the liver and the available results are not clear. There are works showing that TNFα inhibited the suppressive effect of insulin on hepatic glucose production (Lang et al., 1992;

Cheung et al., 1998), nevertheless this inhibition was not observed in another study (Hotamisligil et al., 1993). In view of this, the aim of this work was to investigate the influence of TNFα on the suppressive effect of insulin on HGP and glycogenolysis stimulated by cyclic adenosine monophosphate (cAMP).

MATERIALS AND METHODS

Chemicals

Recombinant rat TNFα was purchased from Peprotech (Mexico SA). Aliquots of reconstituted TNFα were stored at -20°C and thawed immediately before the experiments. Regular insulin was purchased from Eli Lilly (Brazil) and cAMP from Sigma Chemical Co. (St Louis, USA). All other chemicals (98-99.8% purity) were purchased from Sigma Chemical Co. (St Louis, USA), Acros Organics (New Jersey, USA), Reagen (Rio de Janeiro, Brazil) and Merck (Darmstad, Germany).

Animals and administration of TNFα

Male Wistar rats (200-230g), kept at a constant temperature (23±2°C), in a light/dark cycle of 12 h and fed with a standard commercial laboratory diet (Nuvilab®), were used in all experiments.

TNFα (10 µg/Kg) was administered in rats, 1 or 6 h before liver perfusion. The administration consisted of injection, into the penial vein, of 200 µl of a buffered saline solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM, KH_2PO_4 and 0.1% bovine serum albumin), pH 7.4, containing 2 µg TNFα. Control rats were injected with 200 µl of buffered saline. All protocols with animals were approved by the Ethics Committee for Animal Experimentation of the State University of Londrina.

Liver perfusion

For the surgical procedure, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) and submitted to *in situ* liver perfusion, as previously described (Mario et. al, 2009; Leonardo et al., 2009). The perfusion fluid, Krebs-Henseleit buffer (KHB), pH 7.4, at 37ºC and saturated with a 95%:5% O_2:CO_2 mixture, was introduced in the liver (4 mL/min per gram of liver) through a cannula inserted into the portal vein, while a second cannula in the inferior vena cava was used to collect the effluent perfusate. The composition of the KHB buffer was: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 2.5 mM $CaCl_2$. cAMP and insulin were dissolved in KHB. Livers were perfused with KHB during 10 min, with KHB + cAMP (3 µM) between 10-24 min and with KHB + cAMP (3 µM) or KHB + cAMP (3 µM) + insulin (20 µU/mL or 500 µU/mL) between 24-50 min. The effluent perfusate was collected every 2 min to measure the production of glucose, lactate and pyruvate. At the end of the perfusion, the liver was removed and weighed, to allow precise metabolic calculations and the correction of flux rates. Glycogenolysis was calculated as the sum of glucose production plus the half-sum of lactate and pyruvate production [glucose + ½ (lactate + pyruvate)]. The areas under curves (AUCs) of glucose production and glycogenolysis were calculated at interval of 24-50 min.

Analytical procedures

The concentrations of glucose (Bergmeyer and Bernt, 1974), pyruvate (Czok and Lamprecht, 1974) and lactate (Gutmann and Wahlefeld, 1974) were assayed by enzymatic methods previously described.

Statistical procedures

Normal distribution and variance homogeneity were tested and Anova One-Way followed by Newman-Keuls was employed to analyze the results. Statistical analysis was carried out with the program GraphPad Prism 4.0, at the 5% level of significance ($p<0.05$). Data are expressed as mean ± standard error of the mean *(*SEM*).*

RESULTS

Infusion of cAMP (3 μM) in the liver, at 10 min of the perfusion period, stimulated glycogenolysis ($p<0.05$) and increased HGP ($p<0.05$) in all groups. There were no differences in these parameters between the rats administered with TNFα and controls rats (Figures 1, 2, 3 and 4).

Furthermore, infusion of insulin 20 μU/mL or 500 μU/mL in the liver of control rats, at 24 min of the perfusion period, decreased ($p<0.05$) HGP and glycogenolysis stimulated by cAMP, as demonstrated by the respective AUCs (Figures 1, 2, 3 and 4).

In addition, endovenous administration of TNFα, 1 h before liver perfusion, completely abolished ($p<0.05$) the inhibitory effect of insulin 20 μU/mL on HGP (Figures 1A and 1B) and glycogenolysis (Figures 1C and 1D) stimulated by cAMP, as shown by AUCs. However, administration of TNFα, 6 h before liver perfusion, only tended to decrease the inhibitory effect of insulin 20 μU/mL on HGP (Figures 2A and 2B) and glycogenolysis (Figures 2C and 2D) stimulated by cAMP, as revealed by AUCs.

In contrast to the results found with insulin 20 μU/mL, administration of TNFα 1 h before perfusion only tended to reduce the inhibitory effect of insulin 500 μU/mL on HGP (Figures 3A and 3B) and glycogenolysis (Figures 3C and 3D) stimulated by cAMP, as observed by AUCs. Moreover, administration of TNFα 6 h before infusion had no effect on the inhibitory action of insulin 500 μU/mL on HGP (Figures 4A and 4B) and glycogenolysis (Figures 4C and 4D) stimulated by cAMP, as shown by AUCs.

DISCUSSION

The main question of this work was if TNFα acutely influences the hepatic effects of insulin on the catabolism of glycogen stimulated by cAMP. In order to find the answer to this question, TNFα was intravenously administered in rats and after 1 or 6 h the livers were submitted to *in situ* perfusion with cAMP in the presence or absence of physiological (20 μU/mL) or supraphysiological (500 μU/mL) concentrations of insulin. *In vivo* administration of TNFα was used because its direct portal infusion in the liver did not alter HGP from alanine, but endovenous administration of TNFα decreased HGP from this gluconeogenic precursor in rat liver perfusion (Kelmer-Bracht et al., 2006).

Administration of TNFα did not influence the stimulatory effect of cAMP on the catabolism of glycogen, as observed by the similarity in the rates of glycogenolysis and HGP, from 10 to 24 minutes of perfusion, in relation to the control rats (Figures 1, 2, 3 and 4). The stimulatory effects of cAMP on glycogenolysis, and consequently on HGP, were probably mediated by activation of cAMP-dependent protein kinase (PKA) and subsequent phosphorylation and activation of glycogen phosphorylase (Keppens e Wulf, 1975). Accordingly, incubation of isolated rat hepatocytes with TNFα also did not influence glycogenolysis (Rofe et al., 1987).

In control rats, that did not receive TNFα, the stimulatory effects of cAMP on the catabolism of glycogen were reduced in the presence of insulin 20 μU/mL or 500 μU/mL (Figures 1, 2, 3 and 4), confirming the direct inhibitory effect of insulin on perfused liver (Vardanega-Peicher et al., 2003; Borba-Murad et al., 2004). This effect of insulin can be attributed to the reduction of intracellular concentration of cAMP, mediated by activation of phosphodiesterase-3B (PDE3B), an enzyme that promotes hydrolysis and consequent inactivation of cAMP (Pilkis e Granner, 1992; Kitamura et al., 1999). PDE3B is activated by Akt/PKB, via stimulation of PI3K associated with IRS-1 (Zhao et al., 2000).

However, administration of TNFα, 1 h before liver perfusion, completely abolished the inhibitory effect of insulin 20 μU/mL on glycogenolysis and HGP stimulated by cAMP (Figure 1). These results reveal an *in vivo* acute inhibitory effect of TNFα on hepatic response to insulin, decreasing the insulin sensitivity in the catabolism of glycogen. Accordingly, the infusion of TNFα in rats inhibited insulin-induced suppression on HGP (Lang et al., 1992). Furthermore, in obese rats, in which the activity of TNFα was inhibited, HGP was completely suppressed during hyperinsulinemic glucose clamp (Cheung et al., 1998). However, in another study with obese rats the inhibition of TNFα did not affect the suppression of HGP mediated by insulin (Hotamisligil et al., 1993).

Also in agreement with our results, several studies have demonstrated that TNFα inhibited the insulin signaling cascade in the hepatocytes, an effect that may have been due to the activation of serine kinases (Kanety et al., 1995). Indeed, portal infusion of TNFα increased serine phosphorylation of IRS-1 and inhibited the insulin signaling in the liver (Xu et al., 2008). Similarly, incubation of hepatoma cells with TNFα induced serine phosphorylation of IRS-1 (Gupta et al., 2007). Still, incubation of hepatoma cells (Feinstein et al., 1993) or incubation of adipocytes (Miura et al., 1999) with TNFα, for 1 h, decreased tyrosine phosphorylation of IR and IRS-1 stimulated by insulin, indicating that 1 hour is enough time for the induction of the effects of TNFα on the insulin signaling pathway.

A reduction of the effects of insulin on the liver, as promoted by TNFα in the present study, results in increased HGP. Thus, increased production of TNFα may be one of the factors that contributes to the increased HGP in type 2 diabetes, obesity and cancer.

Although the administration of TNFα, 1 h before liver perfusion, completely abolished the inhibitory effect of insulin 20 μU/mL on the catabolism of glycogen, the administration of TNFα, 6 h before perfusion, only tended to decrease the suppressive effect of insulin 20 μU/mL on HGP and glycogenolysis stimulated by cAMP (Figure 2), demonstrating that the effect of TNFα declines over time after its administration, a result that may have been due, among other factors, to its metabolization.

In contrast to the results obtained with physiological concentrations of insulin (20 μU/mL), administration of TNFα, 1 h before perfusion, only tended to reduce the inhibitory effect of supraphysiological concentrations of insulin (500 μU/mL) on HGP and glycogenolysis stimulated by cAMP (Figure 3). Moreover, administration of TNFα, 6 h before perfusion, had no effect on the inhibitory action of insulin 500 μU/mL on the catabolism of glycogen stimulated by cAMP (Figure 4). Therefore, the inhibitory effect of TNFα on the actions of insulin reduced with the increase of insulin concentration infused in the liver. In other words, a supraphysiological concentration of insulin, mimicking the compensatory hyperinsulinemia that occurs in the early stages of insulin resistance associated to type 2 diabetes (Mishima et al., 2001) and obesity (Liang et al., 2008), was necessary to overlap the inhibitory effect of TNFα on hepatic actions of insulin. These results suggest that supraphysiological concentrations of insulin can attenuate the reduction of insulin sensitivity induced by TNFα. Accordingly, it was verified that increasing concentrations of insulin were able to reduce the inhibition mediated by TNFα in the insulin signaling cascade (Hotamisligil et al., 1994). It was also verified that a compensatory hyperinsulinemia, consequent to hypertrophy of pancreatic islets, maintained normoglycemia in an animal model of insulin resistance induced by obesity (Liang et al., 2008). In addition, hepatic ketogenesis, which is inhibited by insulin in normal state, remained suppressed by compensatory hyperinsulinemia in a state of insulin resistance (Wilcox, 2005).

It can be concluded that TNFα decreased the suppressive effect of insulin on HGP and glycogenolysis stimulated by cAMP, 1 h after its administration, suggesting that TNFα may be one of the factors that contributes to the increase of HGP in obesity, type 2 diabetes and cancer. These effects of TNFα were observed only for physiological concentration of insulin, indicating that high concentrations of this hormone can reduce the inhibition of TNFα on the hepatic effects of insulin.

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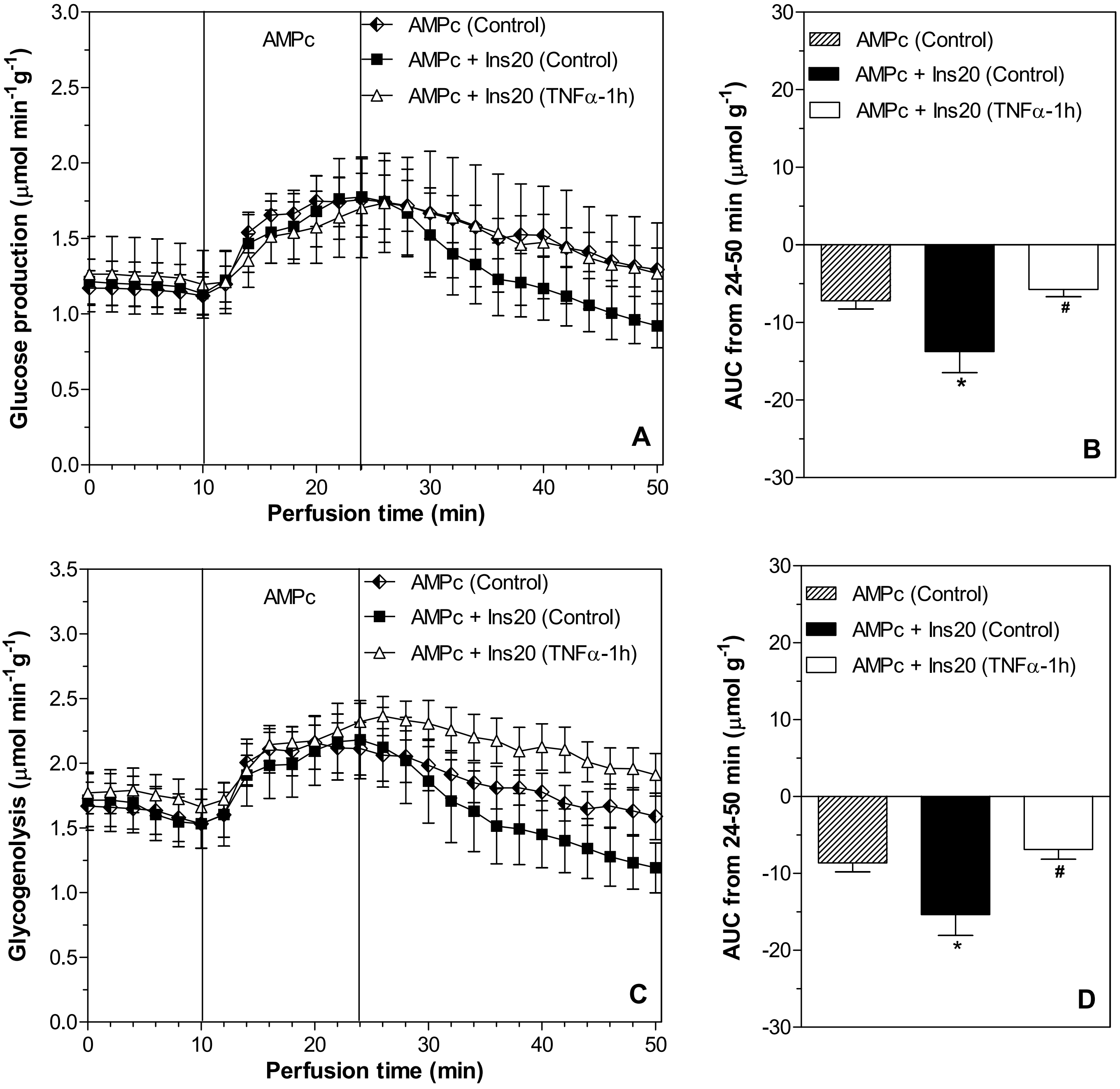

Figure 1 - Effect of tumor necrosis factor α (TNFα) on the suppressive action of insulin on hepatic glucose production (A) and glycogenolysis (C) stimulated by cAMP and the corresponding areas under curves (AUCs) (B, D). Livers were perfused 1 h after administration of 10 μg/Kg TNFα (TNFα-1h) or buffered saline (Control), as described in Materials and Methods. cAMP (3 μM) was infused between 10-24 min. cAMP (3 μM) or cAMP (3 μM) + insulin (20 μU/mL) were infused between 24-50 min. Data are mean ± SEM from 6-9 experiments. $^{*}p<0.05$ *vs* AMPc, $^{\#}p<0.05$ *vs* AMPc + Ins20 (Anova One-Way followed by Newman-Keuls).

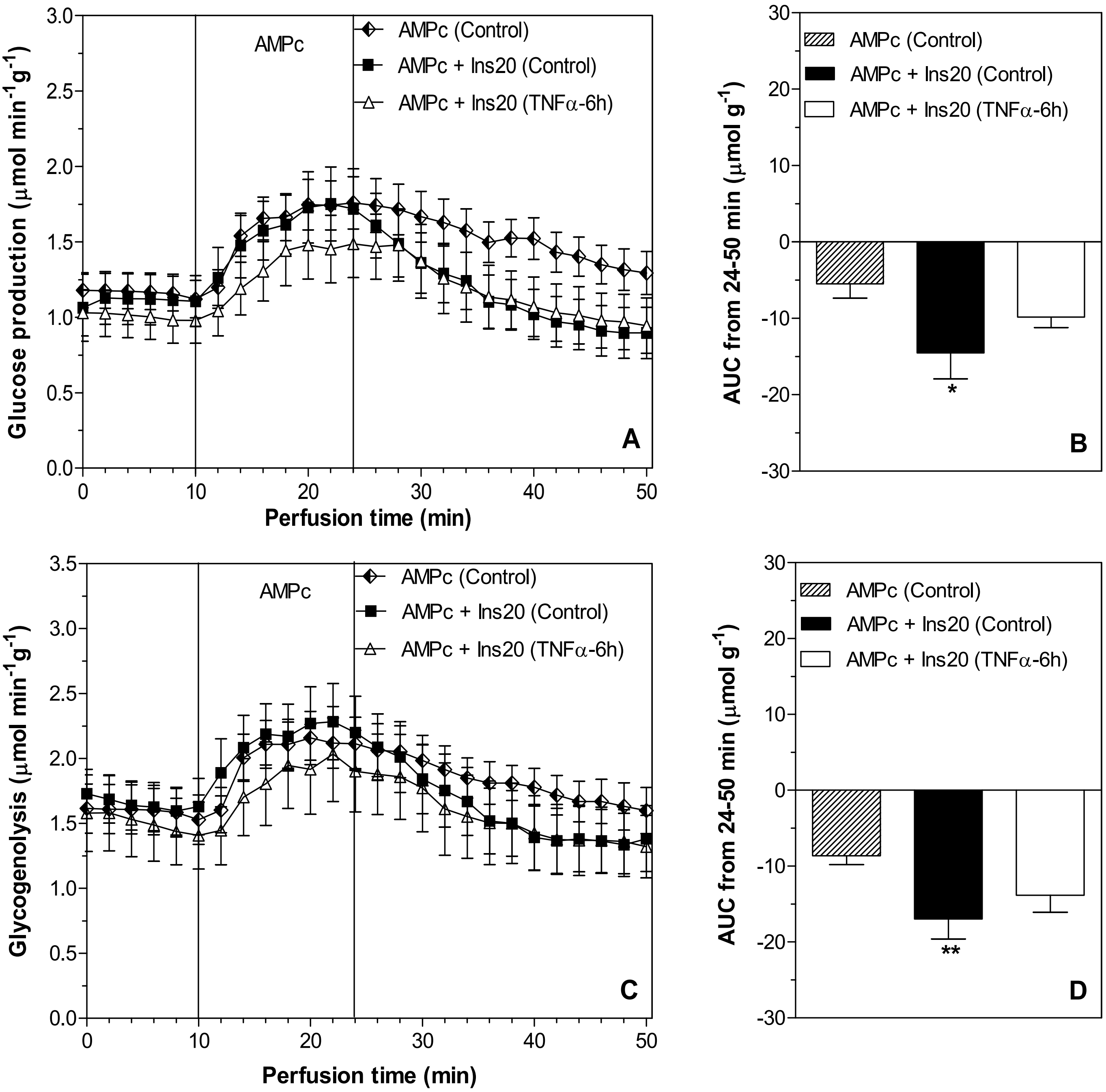

Figure 2 - Effect of tumor necrosis factor α (TNFα) on the suppressive action of insulin on hepatic glucose production (A) and glycogenolysis (C) stimulated by cAMP and the corresponding areas under curves (AUCs) (B, D). Livers were perfused 6 h after administration of 10 μg/Kg TNFα (TNFα-6h) or buffered saline (Control), as described in Materials and Methods. cAMP (3 μM) was infused between 10-24 min. cAMP (3 μM) or cAMP (3 μM) + insulin (20 μU/mL) were infused between 24-50 min. Data are mean ± SEM from 6-7 experiments. *p<0.05 and **p<0.01 *vs* AMPc (Anova One-Way followed by Newman-Keuls).

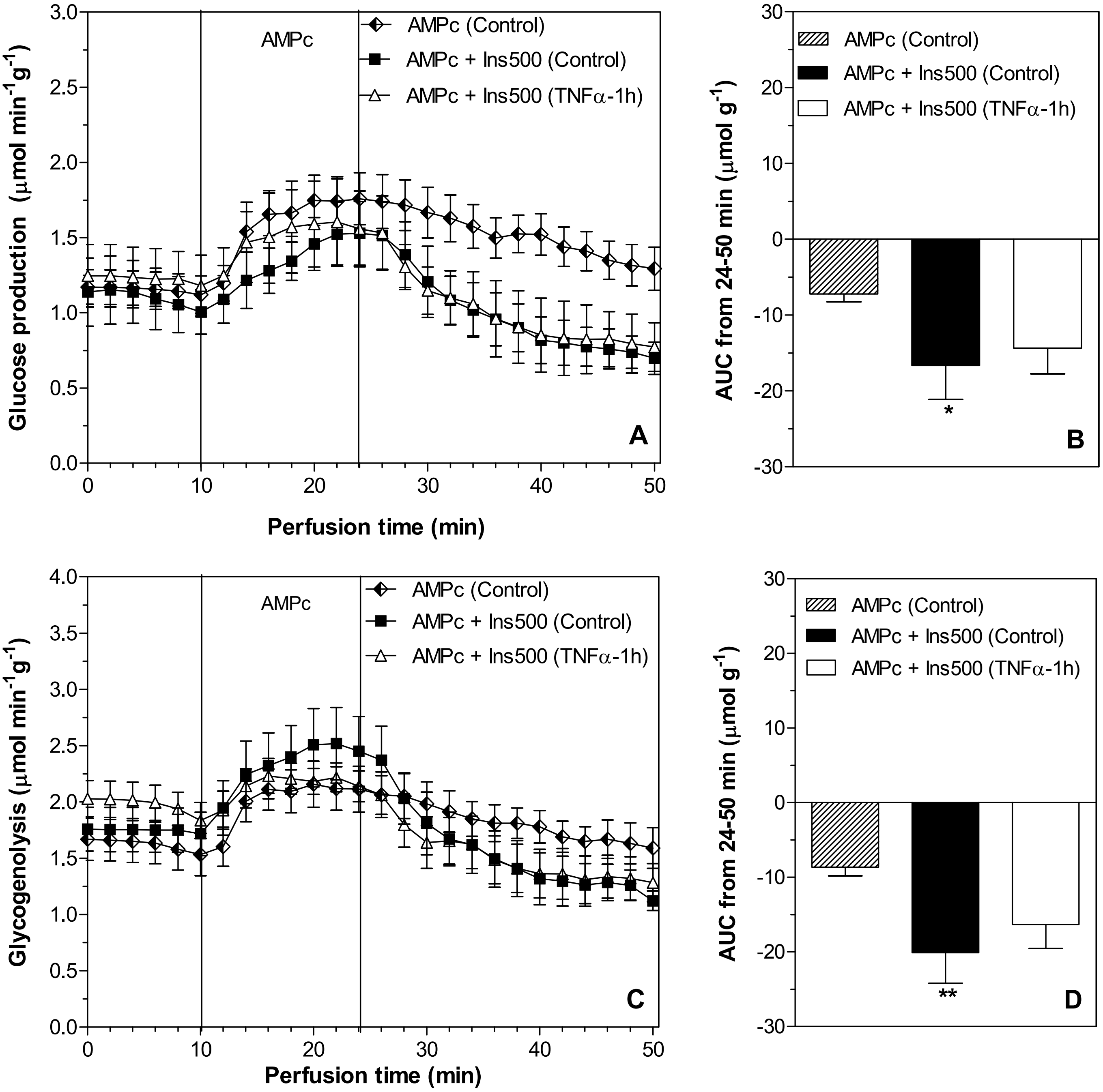

Figure 3 - Effect of tumor necrosis factor α (TNFα) on the suppressive action of insulin on hepatic glucose production (A) and glycogenolysis (C) stimulated by cAMP and the corresponding areas under curves (AUCs) (B, D). Livers were perfused 1 h after administration of 10 μg/Kg TNFα (TNFα-1h) or buffered saline (Control), as described in Materials and Methods. cAMP (3 μM) was infused between 10-24 min. cAMP (3 μM) or cAMP (3 μM) + insulin (500 μU/mL) were infused between 24-50 min. Data are mean ± SEM from 6-7 experiments. *p<0.05 and **p<0.01 *vs* AMPc (Anova One-Way followed by Newman-Keuls).

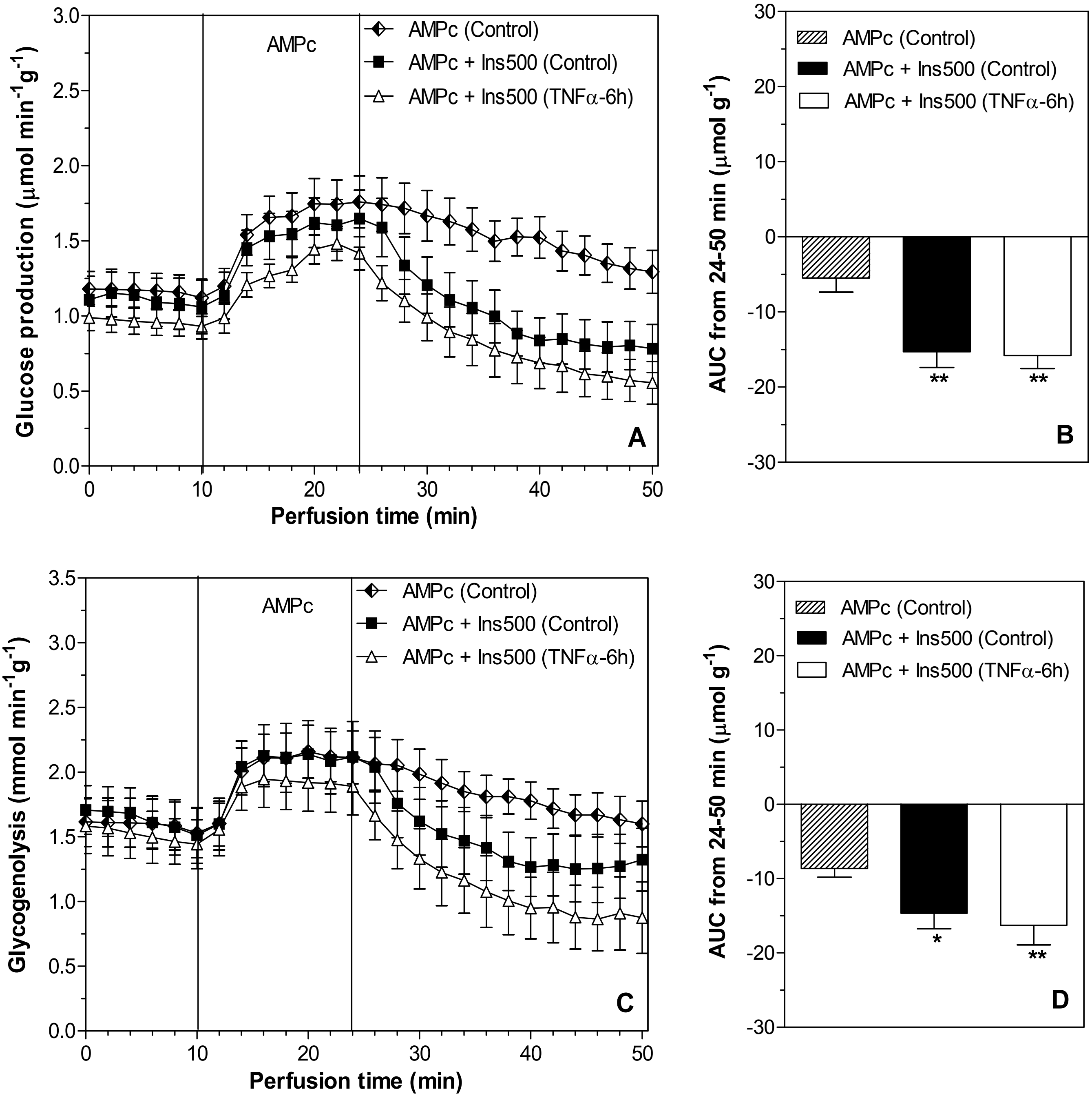

Figure 4 - Effect of tumor necrosis factor α (TNFα) on the suppressive action of insulin on hepatic glucose production (A) and glycogenolysis (C) stimulated by cAMP and the corresponding areas under curves (AUCs) (B, D). Livers were perfused 6 h after administration of 10 µg/Kg TNFα (TNFα-6h) or buffered saline (Control), as described in Materials and Methods. cAMP (3 µM) was infused between 10-24 min. cAMP (3 µM) or cAMP (3 µM) + insulin (500 µU/mL) were infused between 24-50 min. Data are mean ± SEM from 6-7 experiments. *p<0.05 and **p<0.01 *vs* AMPc (Anova One-Way followed by Newman-Keuls).

CONCLUSÕES

CONCLUSÕES

Os resultados do presente estudo permitiram concluir que:

- O TNFα, administrado 6 horas antes da perfusão de fígado, inibiu a produção hepática de glicose a partir dos precursores alanina e lactato, mas não a partir da glutamina e do glicerol, evidenciando um efeito dependente do precursor, do TNFα, na neoglicogênese. Estes resultados reproduzem a inibição da neoglicogênese hepática que foi observada em ratos portadores de tumor Walker-256, sugerindo o envolvimento do TNFα nas alterações da neoglicogênese induzidas pelo tumor.

- O TNFα diminuiu o efeito supressivo da insulina na HGP e glicogenólise estimuladas pelo AMPc, 1 h após a sua administração, um efeito que ocorreu para concentração fisiológica, mas não para concentração suprafisiológica de insulina. Estes resultados sugerem que o TNFα pode ser um dos fatores que contribui para o aumento da HGP na obesidade, no diabetes tipo 2 e câncer e indicam que altas concentrações de insulina podem reduzir a inibição do TNFα nos efeitos hepáticos da insulina.