TALITA ROMANATTO

AÇÃO DO FATOR DE NECROSE TUMORAL ALFA (TNF-α) NO HIPOTÁLAMO - EFEITOS SOBRE EXPRESSÃO PROTÉICA, INGESTÃO ALIMENTAR E CONSUMO DE O_2/PRODUÇÃO DE CO_2

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Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Mestre em Fisiopatologia Médica, área de concentração Medicina Experimental

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CAMPINAS

2006

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Data: 23/02/2006

Aos meus pais, Mauro e Ângela,

Aos meus avós, João (in memoriam) e Ana,

À minha tia Maria José e ao meu irmão Tales.

AGRADECIMENTOS

Em primeiro lugar gostaria de agradecer imensamente a oportunidade que me foi dada de trabalhar nesse grupo de pesquisa! Em especial ao meu orientador, Dr. Lício Velloso, que abriu as portas para que eu pudesse continuar minha formação, meus sinceros agradecimentos pela paciência, seriedade e confiança dispensadas à realização desse trabalho.

Aos meus pais e familiares, por todo apoio, amor e, principalmente, confiança durante mais esses dois anos longe de casa. Sinto muita falta de todos vocês!

Ao meu grande amor, Rodrigo, que esteve presente desde as canulações até a elaboração da aula final, pela paciência, amor e, acima de tudo, pelo companheirismo.

A todos os amigos do Laboratório, Érika Anne, Graziela, Eliana, Juliana, Marciane, Adriana, Joseane, Maria Esméria, Patrícia Prada, Paty Karoll, Vivian, Maria Fernanda, Fernanda, Alessandra, Andressa, Mirian, Raquel, Karen, Rosa, Cristine, Daniela Miti, Daniela Bertelli, Kellen, Eli, Dennys, Brahma, Henrique, Márcio Pereira, Eduardo, Rodrigo, Marcelo, Josenilson, Davi, Marcel, Cafu, Felipe, pelas discussões, pela descontração e momentos compartilhados. Em especial à minha companheira de experimentos, Maristela, que além da pronta disposição em me ajudar, foi também minha professora e hoje grande amiga.

Aos professores Márcio Torsoni e Zé Barreto pelas discussões acadêmicas e agradável convivência e ao Prof. Dr. Mário J. A. Saad por disponibilizar seu laboratório e por seus ensinamentos.

Ao Prof. Dr. Ariovaldo da Cruz Neto, da UNESP de Rio Claro, por disponibilizar o uso de seu laboratório e pela essencial ajuda nos experimentos de respirometria.

Aos técnicos, Luís Janeri e Jósimo, pelo auxílio e disponibilidade.

Ao Márcio (biotério) pela paciência, amizade e exemplar dedicação. O que seria dos nossos ratinhos sem o nosso "*São Márcio*"....!!!

Ao meu irmão Tales e à minha tia Maria José pelas correções e auxílio na parte escrita do trabalho.

Aos meus queridos amigos da UNESP de Rio Claro, Flávio Gabriel, Cadu, Fafá, Fefê, Graziela, Adriana, Roberta, pelo apoio, mesmo que à distância, nos momentos em que mais precisei.

E, é claro, um agradecimento não muito comum, mas muito digno, a todos os meus queridos ratinhos, por suas vidas sacrificadas, sem as quais não seria possível a realização desse trabalho!

A CAPES, CNPq e FAPES, pelo auxílio financeiro.

Meu muito obrigado a todos!

α-MSH	Hormônio estimulador α–melanocítico
AgRP	Peptídeo relacionado ao agouti
Akt	Proteína quinase B
CART	Peptídeo regulado por cocaína e anfetamina
CRH	Hormônio liberador de corticotrofina
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
ERK	Quinase regulada por sinal extra-celular
FOXO	Fator de transcrição da família *foarkhead*
ICV	Intracerebroventricular
IL-1β	Interleucina-1β
IL-6	Interleucina-6
IKK	Quinase do *I kappa B*
IMC	Índice de massa corpórea
IR	Receptor de insulina
IRS-1	Substrato 1 do receptor de insulina
IRS-2	Substrato 2 do receptor de insulina
JAK	Janus quinase
JNK	Quinase da c-Jun
LH	Hipotálamo lateral
MCH	Hormônio concentrador de melanina

NF-kappaB	Fator de transcrição *nuclear factor kappa B*
NPY	Neuropeptídeo Y
PI3K	Fosfatidilinositol 3-quinase
PMSF	Fluoreto de fenilmetil sulfona
POMC	Pro-opiomelanocortina
PVN	Núcleo paraventricular
QR	Quociente respiratório
Rpm	Rotações por minuto
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
SHP-2	SH2-fosfatase
SOCS	Supressor da sinalização de citocinas
STAT	Transdutor de sinal e ativador de transcrição
TNF-α	Fator de necrose tumoral-alfa
TRH	Hormônio liberador de tirotrofina
Tris	Tri (hidroximetil)-aminometano

RESUMO

A insulina e a leptina induzem uma potente resposta anorexigênica e termogênica atuando sobre neurônios do hipotálamo. Evidências recentes sugerem que a resistência à ação desses hormônios deve participar dos mecanismos centrais que favorecem o desenvolvimento da obesidade e sua associação com o diabetes mellitus. O consumo de dieta rica em gordura induz a expressão de proteínas pró-inflamatórias em hipotálamo de ratos, inclusive da citocina TNF-α. O presente estudo avaliou o efeito de diferentes doses intracerebroventriculares (ICV) de TNF-α sobre a ativação da via de sinalização da insulina, a ingestão alimentar e o consumo de O_2/produção de CO_2. Ratos Wistar, machos de oito semanas, foram canulados por via ICV e tratados com diferentes doses de TNF-α, insulina ou salina. A dose mais alta de TNF-α promoveu uma redução de 20% na ingestão alimentar de 12 horas, sendo esta redução inferior àquela produzida pela insulina. Na mesma dose, a citocina promoveu um aumento da temperatura corporal e do quociente respiratório. O TNF-α induziu a ativação de elementos da via pró-inflamatória no hipotálamo, como JNK, p38 e NFκB, o que resultou na transcrição de genes de resposta rápida e indução de proteínas da família SOCS. Com relação à via molecular da insulina, o TNF-α não induziu a ativação de substratos proximais e intermediários, no entanto, mesmo a dose mais baixa foi capaz de ativar elementos distais da via, como ERK e FOXO-1. Cocluindo, o TNF-α exerce um potente efeito anorexigênico e pró-termogênico no hipotálamo de ratos através de mecanismos independentes da ativação de proteínas proximais da via de sinalização da insulina. ERK e FOXO-1, que participam como intermediários em várias outras vias de sinalização, incluindo a via da insulina, são ativados pelo TNF-α no hipotálamo e podem estar envolvidas no efeito anorexigênico e pró-termogênico do TNF-α nesse órgão.

ABSTRACT

Insulin and leptin induce potent anorexigenic and pro-thermogenic responses in specialized neurons of the hypothalamus. Recent evidence suggest that resistance to the action of these hormones play a role in the mechanisms that favor the development of obesity and its common association with type 2 diabetes mellitus. The consumption of a fat-rich diet induces molecular and functional resistance to leptin and insulin in the hypothalamus, which is accompanied by the expression of pro-inflammatory proteins including the cytokine TNF-α. The present study has evaluated the effect of different doses of intracerebroventricular (ICV) TNF-α upon the activation of insulin signal transduction, food ingestion, and the consumption of O_2/ production of CO_2. For that, eight weeks old male Wistar rats were ICV cannulated and treated with saline, insulin or TNF-α. High dose TNF-α promotes a reduction of 20 % of 12 h food intake, which is an inhibitory effect marginally inferior than the one produced by insulin. In addition, TNF-α increases body temperature and respiratory quotient, effects not reproduced by insulin. TNF-α is also capable of activating canonical pro-inflammatory signal in the hypothalamus, inducing JNK, p38, and NF B, which results in the transcription of early responsive genes and induction of proteins of the SOCS family. Finally, TNF-α does not activate signal transduction through early and intermediary elements of the insulin signaling pathway such as IRS-2 and Akt, however, TNF-α, even at low doses, is capable of activating late elements of the insulin signaling pathway such as ERK and FOXO-1. In conclusion, TNF-α exerts potent anorexigenic and pro-thermogenic effects in the hypothalamus through mechanisms independent of the activation of proteins that participate in early and intermediary steps of the insulin signaling pathway. ERK and FOXO-1, which act as late signal transducers for several signaling pathways, including insulin, are activated by TNF-α in the hypothalamus and may participate in the anorexigenic/pro-thermogenic effects of TNF-α in this organ.

1- INTRODUÇÃO

O aumento da prevalência de obesidade em várias regiões do planeta vem se revelando como um dos mais importantes fenômenos clínico-epidemiológicos da atualidade. Fatores como a mudança do hábito alimentar e o estilo de vida sedentário, aliados a determinantes genéticos ainda pouco conhecidos, desempenham um papel relevante na patogênese dessa doença (Kopelman, 2000).

A obesidade constitui importante fator de risco para o desenvolvimento de outras doenças também altamente prevalentes na sociedade moderna tais como diabetes mellitus tipo 2, hipertensão arterial, doença arterial coronariana, dislipidemias, certos tipos de câncer, distúrbios circulatórios, entre outras (Jung, 1997; Kopelman, 2000). Conforme dados obtidos em recentes levantamentos epidemiológicos, há elevada prevalência dessas doenças em várias regiões do planeta. Estima-se que perto de 1 bilhão de adultos em todo o mundo apresentem obesidade (IMC $\geq$ 30 Kg/m^2) ou sobrepeso (IMC entre 25 e 30 Kg/m^2), o que equivale a aproximadamente 28% da população mundial (Kopelman, 2000). Projeções da Organização Mundial de Saúde (OMS) apontam para prevalências maiores que 50% nos Estados Unidos e maiores que 25% no Brasil no ano de 2025 (Kopelman, 2000).

A perda do controle coordenado entre ingestão alimentar e gasto energético parece representar um mecanismo central no desenvolvimento da obesidade (Spiegelman and Flier, 2001). A homeostase entre ingestão e gasto calórico, bem como sua interferência sobre o peso corpóreo dependem de uma complexa relação entre disponibilidade de alimentos, hábitos alimentares, níveis de atividade física, termogênese e níveis sanguíneos e hipotalâmicos de hormônios e substratos envolvidos no gasto e armazenamento de energia (Zhang et al., 1994).

Nos últimos dez anos avanços consideráveis foram obtidos na caracterização dos mecanismos hipotalâmicos de controle da fome e da homeostase energética. Em grande parte, tais avanços foram possíveis graças à identificação do hormônio leptina e do estudo de sua ação no sistema nervoso central (Zhang et al., 1994). Em condições fisiológicas, a expressão e secreção de leptina são tanto maiores quanto maior for o percentual de tecido adiposo no organismo em questão. Entretanto, essa secreção não parece ser contínua e estável, sendo a mesma regulada pela ingestão alimentar, jejum prolongado,

níveis de atividade física, temperatura corporal e níveis circulatórios de alguns hormônios e substratos (Friedman and Halaas, 1998).

Os avanços na caracterização dos mecanismos de ação da leptina no hipotálamo logo revelaram que a transdução do sinal deste hormônio sofre importante controle por vias paralelas de sinalização celular, sendo que, até o presente momento, a insulina se destaca como o principal modulador do sinal da leptina (Moyers, 2005; Tartaglia et al., 1995; Zhang et al., 1994).

A insulina ocupa um papel central na manutenção da homeostase energética e coordena o armazenamento e utilização das moléculas combustíveis no tecido adiposo, fígado e músculo esquelético. O aumento plasmático pós-prandial da concentração de insulina promove a captação de glicose e sua conversão em glicogênio ou triglicérides pelo músculo ou tecido adiposo. Em paralelo, a produção hepática de glicose é inibida como resultado da supressão insulino-dependente da gliconeogênese e glicogenólise, e pelo aumento da síntese de glicogênio (Ruan and Lodish, 2003).

Acredita-se que a resistência central (hipotalâmica) à ação da insulina e da leptina participem da fisiopatologia da obesidade. De acordo com resultados obtidos em estudos anteriores realizados pelo nosso grupo, demonstrou-se que receptores de insulina existem, não apenas no núcleo arqueado (como se acreditava anteriormente), mas também no núcleo paraventricular (Torsoni et al., 2003). O receptor de insulina é uma proteína com atividade tirosina quinase de membrana que depende de substratos intracelulares para transduzir corretamente os sinais da insulina. Dois importantes substratos do receptor, IRS-1 e IRS-2, participam da via de sinalização desse hormônio em vários tecidos do organismo de mamíferos. Recentemente observamos que no hipotálamo, onde a insulina exerce efeito anorexigênico, principalmente o IRS-2 é co-expresso com o receptor de insulina no núcleo arqueado, sendo o IRS-1 expresso predominantemente em hipotálamo posterior (Torsoni et al., 2003).

A leptina e a insulina agem sobre diversas vias de sinalização no hipotálamo reprimindo os circuitos neuronais anabólicos que estimulam a incorporação de alimento e inibindo o gasto energético, ao passo que, simultaneamente, estimulam os circuitos

neuronais catabólicos que inibem a incorporação de alimento e aumentam o gasto energético. Até o presente, duas sub-populações de neurônios foram bem caracterizadas no núcleo arqueado. Uma expressa os neurotransmissores orexigênicos NPY (neuropeptídeo Y) e AgRP (peptídeo relacionado ao agouti), enquanto a outra expressa os neurotransmissores anorexigênicos α-MSH (clivado a partir de POMC) e CART (peptídeo regulado por cocaína e anfetamina) (Flier, 2004).

As conexões de ambos os tipos de neurônios se fazem com duas sub-populações distintas tanto no núcleo paraventricular (PVN) quanto no hipotálamo lateral (LH). No PVN existem neurônios que expressam os neurotransmissores CRH (hormônio liberador de corticotrofina) e TRH (hormônio liberador de tirotrofina) (Schwartz et al., 2000). Ambos os neurotransmissores têm funções anorexigênicas e pró-termogênicas, sendo que o TRH desempenha de forma predominante a função pró-termogênica enquanto CRH desempenha predominantemente a função anorexigênica (Flier, 2004; Schwartz et al., 2000). Por outro lado, no LH também duas sub-populações distintas foram caracterizadas, uma delas expressa a orexina, com papel predominantemente orexigênico, e a outra expressando o MCH (hormônio concentrador de melanina), com papel predominantemente, porém não exclusivamente, anti-termogênico (Flier, 2004; Pereira-da-Silva et al., 2003; Schwartz et al., 2000).

Baixos níveis de insulina ou leptina, observados durante o jejum ou quando o percentual de tecido adiposo está baixo, levam à ativação de neurônios NPY/AgRPérgicos promovendo sinais estimulantes para a produção de orexina e MCH no LH. Como conseqüência, há aumento da fome e diminuição da termogênese. Em contraste, após uma refeição ou quando os estoques de gordura estão repletos, os níveis de insulina e leptina estão elevados ocorrendo uma inibição dos neurônios NPY/AgRPérgicos e estimulação de neurônios POMC/CARTérgicos levando a uma redução na expressão de orexina e MCH no LH e aumento da expressão de CRH e TRH no PVN, o que promove saciedade e aumento da termogênese (Schwartz et al., 2000).

Acredita-se hoje que falhas em alguns dos componentes desse complexo sistema de controle da homeostase energética possam desempenhar um papel importante no desenvolvimento da obesidade e que a resistência hipotalâmica à ação da insulina e leptina

desempenhe um papel central na perda do controle entre ingestão alimentar e gasto energético (Schwartz and Kahn, 1999; Schwartz et al., 2000).

A resistência à insulina pode ser definida como uma resposta subnormal a uma determinada concentração de insulina. Em tecidos periféricos, a resistência à insulina se desenvolve como conseqüência da associação de diferentes fatores, entre os quais encontram-se fatores genéticos, fatores associados a variáveis ambientais como idade, padrão alimentar, estilo de vida sedentário, stress, infecções, entre outros (Stein and Colditz, 2004).

Como discutido anteriormente, a ligação da insulina ao seu receptor promove a ativação de uma série de eventos intracelulares que culminam com o controle da expressão gênica, regulação da atividade de canais iônicos, atividade elétrica de neurônios e controle da produção e liberação de neurotransmissores, entre outros. Do ponto de vista molecular pode se avaliar a ação da insulina através da determinação da atividade de vários elementos que participam da via de sinalização desse hormônio (Araujo et al., 2005; Prada et al., 2005).

Até o presente, quatro mecanismos moleculares distintos de resistência à ação da insulina foram caracterizados: ativação, através da via JAK/STAT, de proteínas da família SOCS (Calegari et al., 2005; Emanuelli et al., 2000; Rui et al., 2002), atividade da proteína tirosina fosfatase SHP-2 (Neel et al., 2003; Zhang et al., 2004), fosforilação em serina de proteínas IRS e do receptor de insulina (Sethi and Hotamisligil, 1999) e nitrosilação de substratos da via de sinalização da insulina (Carvalho-Filho et al., 2005). Estudos desenvolvidos por Aguirre e colaboradores mostraram que, em tecidos periféricos, os níveis elevados de citocinas pró-inflamatórias, observados em infecções, no câncer e na obesidade são responsáveis pela ativação da serina quinase JNK e conseqüente fosforilação em serina dos substratos da via da insulina (Aguirre et al., 2000).

De acordo com um estudo realizado pelo nosso grupo, animais experimentais tratados com dieta hiperlipídica por 16 semanas apresentaram a expressão de proteínas pró-inflamatórias no hipotálamo aumentadas, entre elas algumas citocinas. O fenômeno pró-inflamatório no hipotálamo ativa as vias de sinalização pró-inflamatórias da JNK e

NF-kappaB em neurônios do núcleo arqueado e hipotálamo lateral. Proteínas com atividade serina quinase presentes nessas vias catalisam a fosforilação em serina de importantes participantes da via de sinalização da insulina o que leva a uma resistência molecular à ação desse hormônio no hipotálamo de animais alimentados com dieta hiperlipídica. Entre as citocinas com expressão aumentada estão: TNF-α, IL-1β e IL-6 (De Souza et al., 2005).

O principal efeito dessas citocinas na expressão genética é exercido por fatores de transcrição, os quais se ligam ao DNA e modificam o início da transcrição. Os proto-oncogenes de *c-jun* e *c-fos* codificam proteínas que, em determinadas situações, associam-se para formar um fator de transcrição chamado AP-1, que está implicado na regulação de várias funções celulares, como proliferação, diferenciação, e apoptose. Ambos, Fos e Jun também podem agir independentemente como fatores de transcrição. Eles funcionam como oncoproteínas através da ativação transcricional de genes-chave que codificam proteínas promotoras de crescimento ou pela inibição da transcrição de genes que reprimem o crescimento.

O TNF-α, objeto de estudo desse trabalho, foi inicialmente descrito como uma endotoxina que causava necrose em tumores (Carswell et al., 1975). Mais tarde, descobriu-se que o TNF-α era idêntico à caquexina, uma substância encontrada no sobrenadante de culturas de macrófagos expostos à endotoxinas (Beutler et al., 1985). Classicamente, o TNF-α é descrito como o principal mediador da resposta inflamatória aguda contra bactérias gram-negativas, além de participar da resposta contra outros micro-organismos patogênicos. Além disso, muitos dos fenômenos fisiopatológicos observados durante a sepse são atribuídos ao TNF-α. Estudos mais recentes mostraram ainda que o TNF-α ou caquexina, além de induzir caquexia em animais, atua como uma citocina multifuncional, implicada na inflamação, apoptose ou sobrevivência celular e citotoxicidade. O TNF-α é produzido, não apenas por células do sistema imune, mas também por células do tecido adiposo e possivelmente por outros tecidos diferenciados (De Souza et al., 2005). Uma vez secretado, o TNF-α pode circular numa forma monomérica de 17 kDa ou, mais comumente, numa forma estável homotrimérica de 51 kDa. Esse homotrímero atua, preferencialmente, por meio de dois receptores da família de receptores de TNF-α, o TNF-RI de 55 kDa também chamado de p55 e o TNF-RII de

75 kDa também denominado p75. A ligação do TNF-α a esses receptores induz a transdução do sinal intracelular que pode resultar na ativação de diferentes eventos como regulação de apoptose, indução de genes de resposta imediata e ativação da transcrição de genes de resposta inflamatória, inclusive genes codificadores de outras citocinas como IL-1β, IL-6 e IL-10 (Hotamisligil, 2003).

Nas últimas décadas um interesse maior pelo TNF-α foi estabelecido devido à sua implicação no desenvolvimento da resistência à insulina e ao seu potencial papel como regulador da massa de tecido adiposo (Sethi and Hotamisligil, 1999). Dessa forma, como exposto anteriormente, considerando que os níveis de TNF-α estão aumentados no hipotálamo de animais submetidos à dieta rica em gordura, acreditamos que a caracterização molecular da ação do TNF-α no sistema nervoso central, possa permitir avanços consideráveis na compreensão dos mecanismos fisiopatológicos envolvidos na gênese da obesidade e, ainda, gerar conhecimento útil para o desenvolvimento de novas abordagens terapêuticas para essa doença.

2- OBJETIVOS

Os objetivos do presente trabalho foram:

- Avaliar a capacidade do TNF-α em ativar a sinalização pró-inflamatória e a expressão de proteínas no hipotálamo de ratos;

- Avaliar o efeito de diferentes doses de TNF-α infundidas no hipotálamo, sobre a expressão de proteínas envolvidas na via de sinalização da insulina, sobre a ingestão alimentar e consumo de O_2 / produção de CO_2.

3- CAPÍTULOS

TNF-α activates signal transduction in hypothalamus and modulates the expression of pro-inflammatory proteins and orexigenic/anorexigenic neurotransmitters

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Abbreviations used: CRF/CRH, corticotropin releasing factor/hormone; DIO, diet-induced obesity; ICV, intracerebroventricular; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; JNK, c-Jun kinase; LPS, lipopolysaccharide; SOCS-3, suppressor of cytokine signaling-3; TNF-α, tumor necrosis factor-α.

Abstract

Tumor necrosis factor-α (TNF-α) is known to participate in the wastage syndrome that accompanies cancer and severe infectious diseases. More recently, a role for TNF-α in the pathogenesis of type 2 diabetes mellitus and obesity has been shown. Much of the regulatory action exerted by TNF-α upon the control of energy stores depends on its action on the hypothalamus. In this study, we show that TNF-α activates canonical pro-inflammatory signal transduction pathways in the hypothalamus of rats. These signaling events lead to the transcriptional activation of an early responsive gene and to the induction of expression of cytokines and a cytokine responsive protein such as IL-1β, IL-6, IL-10 and SOCS-3, respectively. In addition, TNF-α induces the expression of neurotransmitters involved in the control of feeding and thermogenesis. Thus, TNF-α may act directly in the hypothalamus inducing a pro-inflammatory response and the modulation of expression of neurotransmitters involved in energy homeostasis.

Running title: TNF-α signaling in hypothalamus

Keywords: Tumor necrosis factor; Inflammation; Cachexia; Obesity; JNK; AP1

Introduction

Much interest has centered the anorexigenic properties of tumor necrosis factor-α (TNF-α), due to its participation in the wastage syndromes that accompany cancer and infectious diseases (Matthys and Billiau 1997; Plata-Salaman 2000; Wong and Pinkney 2004). Both, infection- and LPS-induced anorexia can be reverted by treatment with anti-TNF-α antibodies, with soluble TNF-α receptors or with chemical inhibition of TNF-α synthesis (Haslett 1998; Porter et al. 2000). In addition, tumor-induced anorexia and weight loss can be partially rescued by treatment with a soluble TNF-α receptor that antagonizes TNF-α activity (Torelli et al. 1999).

In recent years, a number of studies have shown that TNF-α and other pro-inflammatory proteins are produced by the adipose tissue during the development of obesity (Katsuki et al. 1998; Schmidt et al. 1999). The increased levels of some of these pro-inflammatory proteins in the blood and in insulin-sensitive tissues play an important role in the induction of insulin resistance, which is a hallmark of type 2 diabetes mellitus and occurs in most obese humans and animals (Hotamisligil et al. 1993; Hotamisligil et al. 1994; Hotamisligil 2003). Interestingly, insulin, acting in concert with leptin, provides the most robust adipostatic and anorexigenic signals to the hypothalamus (Schwartz et al. 2000; Carvalheira et al. 2001; Flier 2004), and a phenomenon of hypothalamic resistance to leptin and insulin has been demonstrated in different animal models of obesity (Carvalheira et al. 2003; Torsoni et al. 2003; Howard et al. 2004). In a recent study, we showed that, in diet-induced obesity (DIO), the expression of TNF-α and other pro-inflammatory proteins is remarkably increased in the hypothalamus of rats (De Souza et al. 2005). The inhibition of TNF-α signaling in these animals reduces caloric intake, promotes body weight loss and reverts hypothalamic resistance to insulin (De Souza et al. 2005).

Based on cancer, infection and, more recently, on obesity studies, we can assume that TNF-α and other pro-inflammatory cytokines play important roles in the control of body energy stores. In some cases they participate in energy wastage, whilst in others they take part in body weight gain. Much of this modulatory function

seems to be dependent on cytokine signaling in the hypothalamus. However, little is known about the signaling pathways involved in TNF-α action in the hypothalamus and on the control exerted by this cytokine upon local protein and neurotransmitter expression. Therefore, the objective of this study was to evaluate the property of TNF-α to activate pro-inflammatory signaling and protein expression in the hypothalamus of rats.

Materials and methods

Antibodies, chemicals and buffers

Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA, USA). HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, bovine serum albumin (fraction V), and angiotensin II were from Sigma (St. Luis, MO, USA). Protein A-Sepharose 6MB was from Pharmacia (Uppsala, Sweden), ^{125}I-protein A was from ICN Biomedicals (Costa Mesa, CA, USA), and nitrocellulose paper (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Sodium thiopental (Amytal) and human recombinant insulin (Humulin R) were from Lilly (Indianapolis, IN, USA). Mouse recombinant TNF-α was from Calbiochem (Darmstadt, Germany). The anti-TNF-α monoclonal antibody, Infliximab, was from Centocor (Horsham, PA, USA). Anti-phospho-JNK (mouse monoclonal, recognizing JNK phosphorylated at Thr 183 and Tyr 185, sc-6254), -phospho-p38 (mouse monoclonal, recognizing p38 phosphorylated at Tyr 182, sc-7973), -c-Fos (rabbit polyclonal, sc-7202), -c-Jun (rabbit polyclonal, sc-1694), -IL-1β (rabbit polyclonal, sc-7884), -IL-6 (goat polyclonal, sc-1265), -IL-10 (goat polyclonal, sc-1783), -SOCS-3 (rabbit polyclonal, sc-9023) and CRF/CRH (corticotropin-releasing factor/hormone) (rabbit polyclonal, sc-10718) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Experimental animals

Eight-week old male Wistar rats (*Rattus norvegicus*) from the University of Campinas Central Animal Breeding Center were used in the experiments. The rats were allowed access to standard rodent chow and water *ad libitum*. Food was withdrawn 12 h before the experiments. All experiments were conducted in accord with the principles and procedures described by the NIH Guidelines for the Care and Use of Experimental Animals and were approved by the State University of Campinas Ethical Committee. In all experiments the rats were ICV-cannulated and submitted to treatment with TNF-α (accompanied or not by Infliximab or insulin, according to the protocols described below).

ICV cannulation

All rats were stereotaxically instrumented using a Stoelning stereotaxic apparatus, according to a method previously described (Torsoni et al. 2003). Cannula efficiency was tested 1 w after cannulation by the evaluation of the drinking response elicited by ICV angiotensin II (Johnson and Epstein 1975).

Protocol for food ingestion determination

ICV cannulated rats were food deprived for 6 h (from 12 to 18 h) and at 18 h were ICV treated with insulin (2.0 μL, 10^{-6}mol/L), TNF-α (2.0 μL, 10^{-8} mol/L) or saline (2.0 μL). Food ingestion was determined over the next 12 h.

Tissue extraction, immunoblotting and immunoprecipitation

ICV cannulated rats were anesthetized and acutely treated with saline (2.0 μL) or TNF-α (in most experiments the dose of TNF-α was 10^{-8} mol/L, 2.0 μL, except in dose response experiments when the concentration ranged from 10^{-12} to 10^{-6} mol/L).

Some rats were pre-treated with Infliximab (2.0 μL, 10 mg/mL, ICV) one hour before TNF-α injection. After 10 min, as determined by time-course experiments, the hypothalami were obtained and immediately homogenized in solubilization buffer at 4ºC [1% Triton X-100, 100 mmol/L Tris-HCl (pH 7.4), 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium orthovanadate, 2.0 mmol/L PMSF and 0.1 mg aprotinin/mL] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY). Insoluble material was removed by centrifugation for 20 min at 9,000 x *g* in a 70.Ti rotor (Beckman) at 4ºC. The protein concentration of the supernatants was determined by the Bradford dye binding method. Aliquots of the resulting supernatants containing 2.0 mg of total protein were used for immunoprecipitation with antibodies against c-Fos at 4ºC overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes and blotting with anti-c-Jun. In direct immunoblot experiments, 0.2 mg of protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-phospho-JNK, -phospho-p38, -c-Fos, -c-Jun, -IL-1β, -IL-6, -IL-10, -SOCS-3 and -CRF/CRH antibodies, as described (Araujo et al. 2005).

Plasmid transfection and reporter gene assay

A plasmid containing the positions –250 to +150 of the *c-jun* promoter fused to the firefly luciferase gene (pJC6GL3) was kindly donated by Dr. Ron Prywes (Columbia University, New York, NY, USA). ICV cannulated rats received 0.1 μg of internal control SV40-renila luciferase (Invitrogen, Carlsbad, CA, USA) with or without 2.0 μg of pJC6GL3 and, after six days, the rats were ICV treated with saline (2.0 μL) or TNF-α (10^{-8}M, 2.0 μL). After six hours the hypothalamus was obtained and a protein extract was prepared and used for determination of luciferase activity according to the recommendations of the manufacturer (Dual-Luciferase Reporter Assay System, Promega). This protocol has been previously optimized (Nadruz et al. 2005).

RNA preparation for RT-PCR

Total hypothalamic RNA was extracted using Trizol (Life Technologies, Gaithersburg, MD, USA) reagent, according to the manufacturer's recommendations. Total RNA was rendered genomic DNA-free by digestion with RNAse-free DNAse (RQ1; Promega, Madison, Wisconsin).

Semiquantitative RT-PCR

Seven micrograms of total RNA were reverse-transcribed with SuperScript reverse transcriptase (200 U/μL) using oligo (dT) (50 mmol/L) in a 30 μL reaction volume (5x RT buffer, 10 mmol/L dNTP, and 40 U/μL RNAse free inhibitor). The reverse transcriptions involved a 50 min incubation at 42ºC and a 15 min incubation at 70ºC. The PCR products were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide and visualized by excitation under UV light. Photo-documentation was performed using the Nucleovision System (NucleoTech, San Mateo, CA, USA) and band quantification was performed using the Gel Expert Sofware (NucleoTech). In all samples, the amplification of RPS-29 was performed and used as an internal control for quantity and quality. The semiquantitative expression (SE) of NPY was calculated using the formula: SE=pixel area of product/pixel area of RPS-29 x 100. The primers used, and the PCR conditions were: RPS-29 (NCBI: NM012876), sense: 5' AGG CAA GAT GGG TCA CCA GC 3', antisense: 5' AGT CGA ATC ATC CAT TCA GGT CG 3' (fragment: 202pb; TM: 57ºC; amplification 27 cycles); MCH - melanin concentrating hormone (NCBI: M29712), sense: 5' TAC GGA GCA GCA AAC A 3', antisense: 5' ACA GCC AGA CTG AGG G 3' (fragment: 323pb, TM: 55ºC, amplification 25 cycles); POMC – proopiomelanocortin (NCBI: AF510391), sense: 5' CTC CTG CTT CAG ACC TCC AT 3', antisense: 5'TTG GGG TAC ACC TTC ACA GG 3' (fragment: 398pb, TM: 63ºC, amplification 32 cycles); NPY - neuropeptide Y (NCBI: NM012614), sense: 5' AGAGATCCAGCCCTGAGACA 3', antisense: 5' AACGACAACAAGGGAAATGG 3' (fragment: 236pb , TM: 62ºC, amplification: 31 cycles).

Statistical analysis

Specific protein bands present on the blots were quantified by densitometry. Mean values +/- SEM obtained from densitometric scans were compared utilizing Student's *t*-test for paired samples or by repeat-measures analysis of variance (one-way or two-way ANOVA) followed by *post hoc* analysis of significance (Bonferroni test) when appropriate. A $p<0.05$ was accepted as statistically significant.

Results

TNF-α activates pro-inflammatory signal transduction and early-inducible gene expression in hypothalamus

To establish the optimal dose and time for TNF-α to activate pro-inflammatory signaling in hypothalamus, ICV-cannulated rats were treated with 2.0 μL 10^{-8} mol/L TNF-α and after times ranging from 0 to 30 min the hypothalamus was obtained. In parallel studies, 2.0 μL TNF-α, in concentrations ranging from 10^{-12} to 10^{-6} mol/L, were ICV-injected and the hypothalamus was obtained after 10 min for use in immunoblot experiments. As shown in Figures 1A and 1B, TNF-α promoted maximum phosphorylation of JNK at the dose of 10^{-8} mol/L and at the time of 10 min. TNF-α was also able to produce the phosphorylation of p38 at the optimal time of 10 min (Fig. 1C).
To evaluate the effect of TNF-α on the activation of a pro-inflammatory transcription factor, ICV-cannulated rats were treated with 2.0 μL 10^{-8} mol/L TNF-α and, after the times ranging from 0 to 180 min, the hypothalamus was obtained and used in immunoprecipitation assays with anti-c-Fos antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-c-Jun antibody. AP-1 complex formation started as early as 5 min, reaching a peak at 15 min and returning to basal levels at 60 min. Finally, ICV injected TNF-α (2.0 μL, 10^{-8} mol/L) was able to induce hypothalamic c-Fos (Fig. 1E) and c-Jun (Fig. 1F) expression, which was detectable at 60 min for c-Fos, and started at 15 min, increasing progressively until 60 min for c-Jun. To test the specificity of the signaling events

described above, ICV-cannulated rats were treated with the anti-TNF-α monoclonal antibody Infliximab and after 60 min with TNF-α (2.0 μL, 10^{-8} mol/L). The pre-treatment with Infliximab completely inhibited TNF-α-induced activation of JNK (Fig. 2A) and p38 (Fig. 2B).

TNF-α activates gene transcription through AP-1 in hypothalamus

To examine whether acute TNF-α treatment induces transcriptional regulation of *c-jun,* ICV-cannulated rats were transfected with the plasmid pJC6GL3 containing the *c-jun* promoter fused to the firefly luciferase gene (Fig. 3A). As depicted in Figure 3B the treatment with TNF-α promoted a 30-fold increase in the luciferase activity in the hypothalamus at 6 min.

TNF-α induces the expression of cytokines and SOCS-3 in hypothalamus

To evaluate the ability of TNF-α to modulate cytokine and cytokine responsive protein expression in hypothalamus ICV-cannulated rats were treated with a single dose of TNF-α (2.0 μL, 10^{-8} mol/L) and after the times ranging from 0 to 180 min the hypothalami were obtained and used in immunoblotting experiments. As depicted in Figure 4, TNF-α induced the hypothalamic expression of IL-1β starting at 15 min and increasing continuously up to 180 min (Fig. 4A). TNF-α also induced the expression of IL-6 starting at 15 min and increasing continuously up to 180 min (Fig. 4B), and of IL-10 starting at 15 min and increasing continuously up to 180 min (Fig. 4C). Finally, TNF-α was able to induce the expression of the inhibitor of cytokine signaling, SOCS-3, which was detected at 120 and 180 min (Fig. 4D).

TNF-α inhibits food intake and modulates orexigenic/anorexigenic neurotransmitter expression in hypothalamus

The effects of acute treatment with TNF-α were evaluated upon the expression of two orexigenic (NPY and MCH) and two anorexigenic (POMC and CRH) neuropeptides. TNF-α induced the expression of NPY (Fig. 5A), POMC (Fig. 5B) and MCH (Fig. 5C) mRNAs starting at 15 min and lasting for at least 120 min. TNF-α also induced CRF/CRH protein expression (Fig. 5D), beginning at 120 min. Of note, is the finding that the TNF-α-induced increase in the orexigenic neurotransmitter levels reached a maximum of 1.3-fold, while the increase in anorexigenic neurotransmitter levels reached 1.8-fold for CRF/CRH and 8.0-fold for POMC. Finally, the acute effect of TNF-α on spontaneous food intake was determined and compared with the effect of insulin. As depicted in Fig. 5E, insulin promoted a 45% reduction in 12-h food intake while TNF-α induced a 25% reduction in feeding during the same time frame.

Discussion

TNF-α signal transduction is dependent on ligand binding to at least two distinct receptors, TNF-R1 and TNF-R2 (Locksley et al. 2001). TNF-R2 is expressed mostly in cells of the immune system and is thought to play an important role in the ontogenesis and differentiation of lymphoid tissues (Locksley et al. 2001), whilst TNF-R1 is widely distributed and mediates TNF signaling towards activation of pro-inflammatory responses, control of gene expression, and regulation of apoptosis, amongst a number of other functions (Wajant et al. 2003).

In cells of the immune system and in other tissues the intracellular outcomes of TNF-α signaling have been thoroughly studied and the capacity of this cytokine to activate signal transduction through NF-kappaB, JNK, p38 and intermediaries of apoptosis is well known. However, in the central nervous system and more specifically in the hypothalamus, little is known about the transduction of the TNF-α signal (Owens et al. 2005).

Here, we have evaluated TNF-α signal transduction and activation of gene expression in the hypothalamus of rats. We believe that most, if not all of the effects of TNF-α determined in this study were dependent on TNF-R1, since TNF-R2 is almost absent in the brain of rodents not exposed to any pro-inflammatory or growth-inducing stimulus (Bette et al. 2003). In the first part of the study, we show that an acute ICV injection of TNF-α is capable of inducing the activation of JNK and p38. These events were accompanied by the induction of AP-1 and the expression of c-Jun and c-Fos. We also show that these effects are specifically dependent on TNF-α since the pre-treatment of rats with the anti-TNF-α monoclonal antibody, Infliximab, completely prevented the activation of signal transduction. Finally, by evaluating the outcomes of ICV TNF-α treatment upon the activation of the transfected construct containing the *c-jun* promoter fused to the firefly luciferase gene, we provide strong evidence for the integrity of the TNF-α signaling system, from membrane to nucleus, in cells of the hypothalamus.

No previous study has looked into JNK and p38 engagement by TNF-α in the hypothalamus. In other regions of the central nervous system and in brain cell cultures TNF-α signaling has been shown to induce the activation of JNK and p38 in conditions such as acute stimulation with TNF-α, experimental trauma and viral infections (Zhang et al. 1996; de Bock et al. 1998; Ladiwala et al. 1999). In most cases, TNF-α or TNF-α-inducing stimuli promote an early activation of JNK and p38 signaling in a pattern very similar to the one observed here in the hypothalamus. Thus, we can assume that the hypothalamus of rats is fully equipped with the molecular apparatus required for TNF-α signal transduction through JNK and p38.

We next evaluated the effects of TNF-α upon the expression of two pro-inflammatory cytokines (IL-1β and IL-6) and one anti-inflammatory (IL-10) cytokine and of a cytokine responsive protein, SOCS-3, in the hypothalamus. As a rule, all three cytokines were rapidly induced in response to TNF-α, starting as early as 15 min and increasing continuously to up to 3 h. In cell culture systems (Sawada et al. 1992; Norris et al. 1994; Sheng et al. 1995) and in different regions of the brain (Bristulf and Bartfai 1995; Pitossi et al. 1997; Zhai et al. 1997; Didier et al. 2003) TNF-α has been

shown to induce the expression of cytokines. In general, the response to TNF-α stimulus is rapid, suggesting that this is a primary event. This seems to be the case here. We believe that, in our experiments, the induction of IL-1β, IL-6 and IL-10 were all primarily dependent on TNF-α and required no intermediaries. With regard to SOCS-3, it was initially thought that its expression would be controlled exclusively by cytokine signaling through type I cytokine receptors (Auernhammer and Melmed 2001). More recently, it was realized that SOCS-3 could be induced also by cytokine and hormone signaling through other classes of receptors, including TNF-α (Bjorbaek et al. 1998; Emanuelli et al. 2001; Rui et al. 2002; Calegari et al. 2003; Torsoni et al. 2004). The capacity of TNF-α to induce SOCS-3 was shown in adipose tissue (Emanuelli et al. 2001), in liver (Campbell et al. 2001; Sass et al. 2005), and in cells of the immune system (Bode et al. 2003). However, no previous study has demonstrated the capacity of TNF-α to induce SOCS-3 (or other SOCS family members) in neural tissues. Due to the moderately long time frame required for TNF-α to induce SOCS-3 in our study, we cannot be sure if this is a primary event or if it depends on the expression of intermediaries. It is of particular interest that IL-6 has been shown to induce SOCS-3 in the brain (Lebel et al. 2000), however, in this case, and also in most other situations that lead to the induction of SOCS-3, there is a requirement for a moderately long time for the protein to be detected. Thus, at this point we can assume that TNF-α is capable of inducing SOCS-3 expression in hypothalamus but whether this is a primary or a secondary event remains to be determined.

In the last part of the study, we evaluated the capacity of TNF-α to modulate neurotransmitter expression in the hypothalamus. Using RT-PCR or immunoblot, we observed that both orexigenic and anorexigenic peptides were significantly and rapidly induced by TNF-α treatment. Due to the involvement of TNF-α in the cachexia syndrome that accompanies cancer and some infectious diseases, much interest has been drawn upon the ability of TNF-α to modulate the expression of hypothalamic peptides involved in energy homeostasis, however, in most studies the effects of TNF-α were evaluated indirectly. In general, in most systems tested, TNF-α was shown to be capable of inducing increases in the expressions of CRF/CRH (Bernardini et al. 1990; Watanobe and Takebe

1992), and POMC/MSH (Wisse et al. 2003). With respect to NPY, most studies could not detect any significant changes in its expression in response to TNF-α (King et al. 2000), however, the effect of TNF-α, injected directly in the hypothalamus has not been tested in any study. Finally, we could find no study that has evaluated the direct effect of TNF-α on MCH expression. It is interesting to note that, in the present study, although we detected significant increases in expression of both orexigenic and anorexigenic neurotransmitters, the effect upon the anorexigenic peptites was of a remarkably higher magnitude. Thus, we believe that the balance of neuropeptide expression favoring anorexigenic inputs may have an impact on the outcome of TNF-α action in the hypothalamus, which is clearly anorexigenic in the doses used in this study.

In conclusion, this study shows that TNF-α can activate a cascade of signal transduction in the hypothalamus of rats. These signaling events induce the expression of cytokines and other proteins related to inflammatory signaling that, on a long-term basis, may have implications in the local response to a pro-inflammatory stimulus. In addition, TNF-α can, per se, modulate the expression of neurotransmitters involved in the control of energy homeostasis, favoring energy wastage.

Acknowledgements

We thank Mr. L. Janeri for technical assistance. The costs for these studies were defrayed by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank Dr. Nicola Conran for English language editing.

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Legends for the figures

Figure 1- TNF-α-induced signal transduction and activation of early responsive gene expression in the hypothalamus. Anesthetized rats were ICV treated with 2.0 μL, 10^{-8} mol/L TNF-α (A, C-F) or at the concentrations depicted in the figure (B). After the time frames, as depicted in the figures (A, C-F) or after 10 min (B), hipothalami were obtained for protein extract preparation. For direct immunoblot analysis (A-C, E, F), samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific antibodies to phospho-JNK (p-JNK) (A, B); phospho-p38 (p-p38) (C); c-Fos (E) and c-Jun (F). For immunoprecipitation followed by immunoblot, samples containing 2.0 mg total protein were submitted to immunoprecipitation (IP) with antibodies to c-Fos (D); immunoprecipitates were collected with Protein A-Sepharose and separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with antibodies to c-Jun. Specific bands were quantified by densitometric analysis. In all experiments, n = 4; *$p<0.05$ *vs.* time 0 (A, C-F) or *vs.* concentration 0 (B). Results are presented as mean ±SEM.

Figure 2- Specificity of the TNF-α-induced signal transduction in the hypothalamus. Anesthetized rats were ICV treated with 2.0 μL, 10^{-8} mol/L TNF-α (TNF-α) or pre-treated with Infliximab 2.0 μL, 10 mg/mL and after one hour with 2.0 μL, 10^{-8} mol/L TNF-α(I + T) . After 10 min, hipothalami were obtained for protein extract preparation. Samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific antibodies to phospho-JNK (p-JNK) (A) or phospho-p38 (p-p38) (B). Specific bands were quantified by densitometric analysis. In all experiments, n=4; *$p<0.05$ *vs.* control (C) (treated ICV with similar volume of saline). Results are presented as mean ±SEM.

Figure 3- Activation of the *c-jun* promoter by TNF-α in the hypothalamus. The schematic representation of the *c-jun* promoter fused to the firefly luciferase gene is depicted in A. In B, ICV cannulated rats were treated with 0.1 μg internal control SV40-renila luciferase without (C) or with (*c-jun*) 2.0 μg the pJC6GL3 construct containing the *c-jun* promoter. After 6 days, rats were treated with a single dose of TNF-α (10^{-8} mol/L, 2.0 μL) and after 6 h hipothalami were obtained for determination of luciferase activity. In all experiments, n=4; *p<0.05 *vs.* control (C). Results are presented as mean ±SEM.

Figure 4- TNF-α induces the expression of cytokines and a cytokine responsive protein in hypothalamus. Anesthetized rats were ICV treated with 2.0 μL, 10^{-8} mol/L TNF-α and after the time frames depicted in the figure, the hipothalami were obtained for protein extract preparation. Samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific antibodies to IL-1β (A); IL-6 (B); IL-10 (C) or SOCS-3 (D). Specific bands were quantified by densitometric analysis. In all experiments, n=4; *p<0.05 *vs.* control (0). Results are presented as mean ±SEM.

Figure 5- TNF-α regulates hypothalamic neurotransmitter expression and food intake. Anesthetized rats were ICV treated with 2.0 μL, 10^{-8} mol/L TNF-α and, after the time frames depicted in the figure the hipothalami were obtained for RNA (A-C) or protein extract (D) preparation. In A-C RT-PCR reactions were conducted according to the settings presented in Materials and methods section. The amounts of NPY (A); POMC (B); or MCH (C) were calculated as a fraction of the internal control RPS-29. In D, samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with a specific antibody to CRF/CRH. Specific bands were quantified by densitometric analysis. In E, ICV cannulated rats were food deprived for 6 h (from 12 to 18 h) and at 18 h were ICV treated with insulin (2.0 μL, 10^{-6}mol/L), TNF-α (2.0 μL, 10^{-8} mol/L) or saline (C) (2.0 μL). Food ingestion was determined over the next 12 h. In all experiments, n=4; *p<0.05 *vs.* control (0) (A-D) and (S) (E). Results are presented as mean ±SEM.

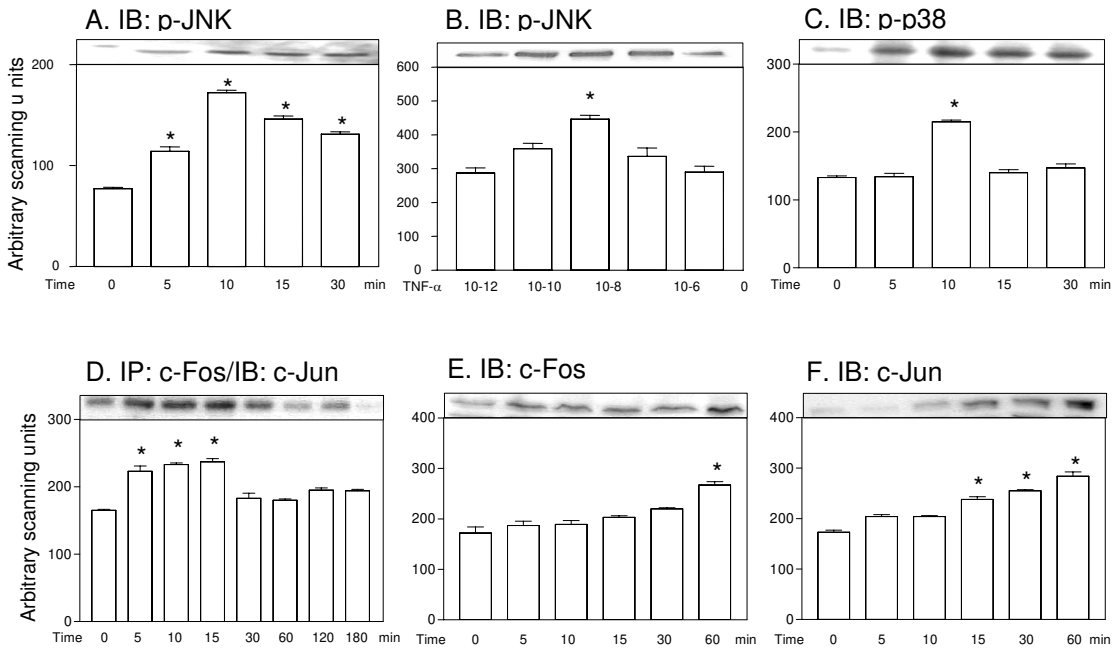


Figure 1

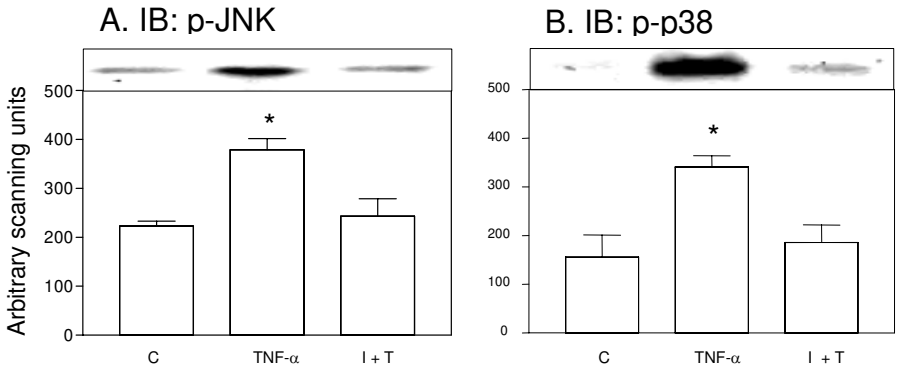


Figure 2

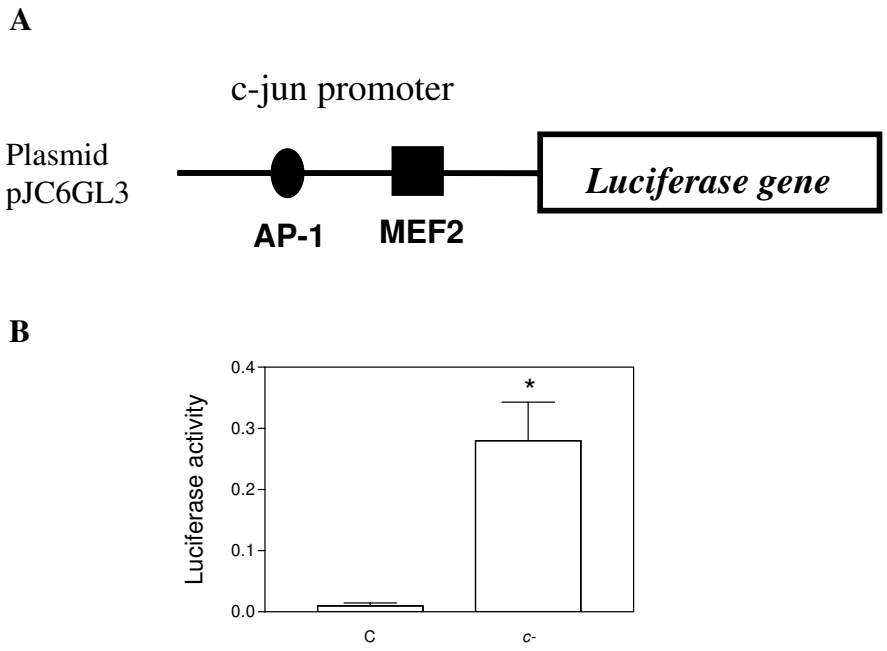


Figure 3

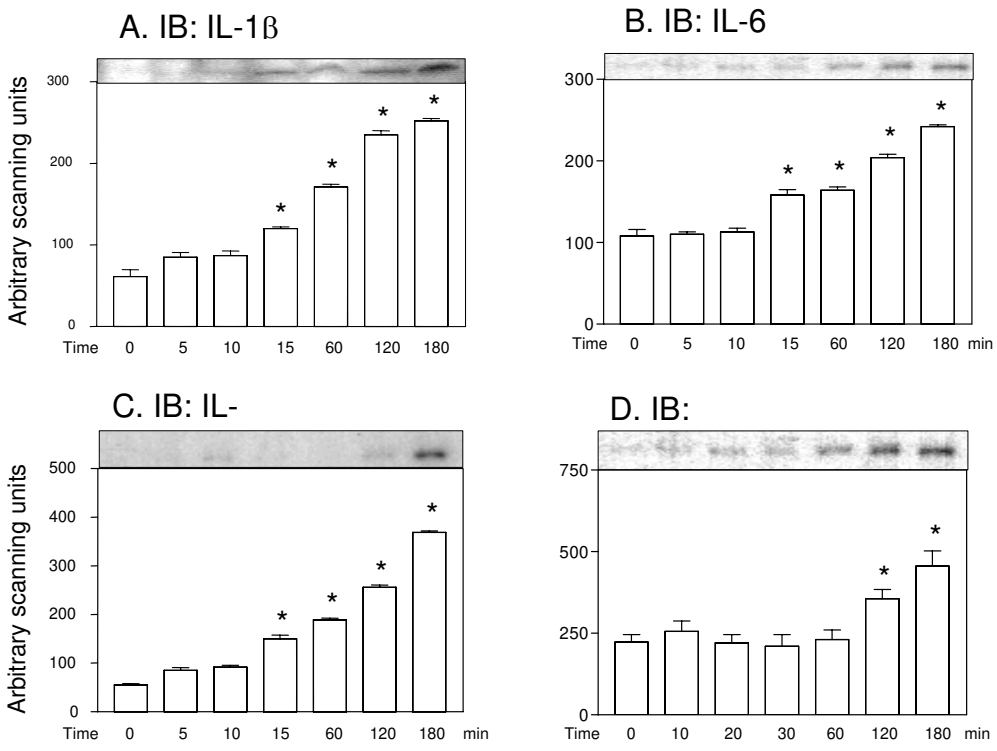
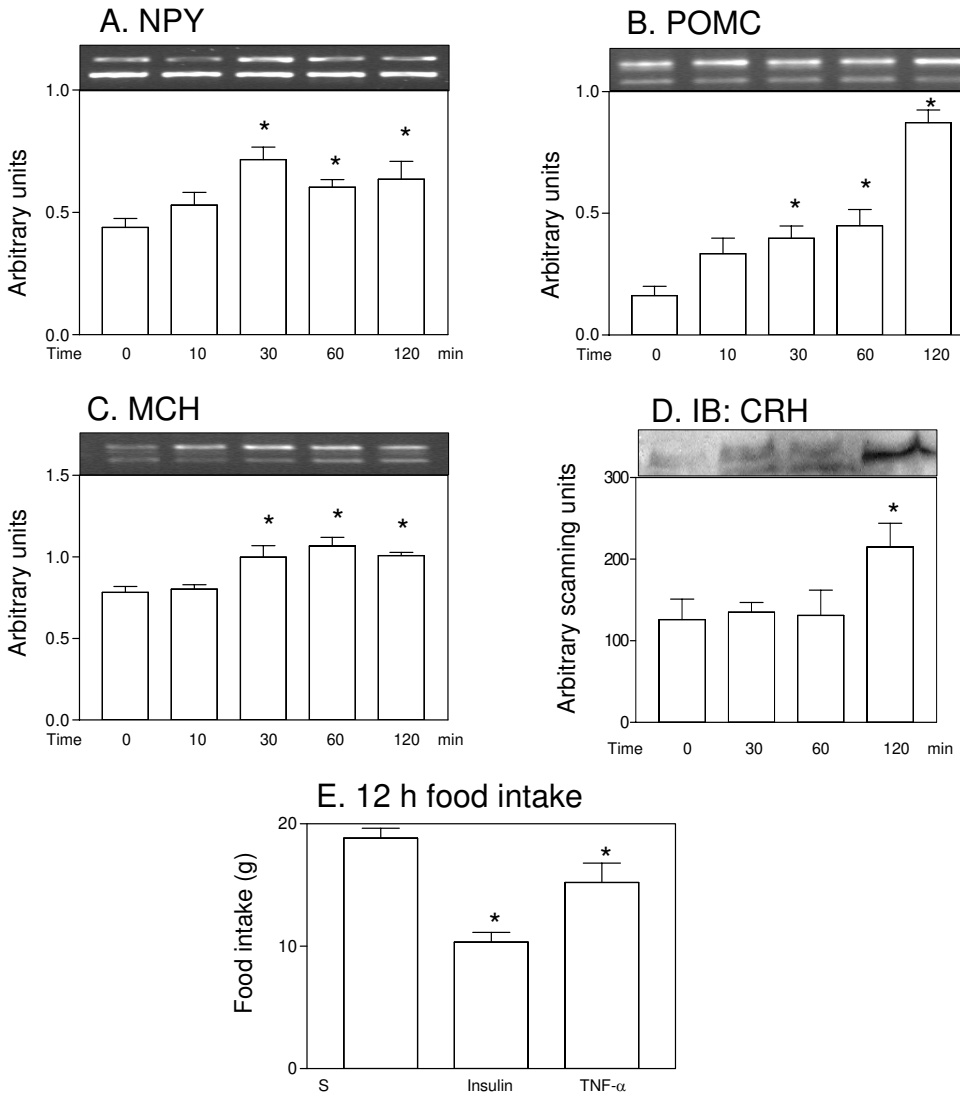


Figure 4


A. NPY
Arbitrary units
1.0
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Time 0 10 30 60 120 min
B. POMC
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Time 0 10 30 60 120
C. MCH
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1.5
1.0
0.5
0.0
Time 0 10 30 60 120 min
D. IB: CRH
Arbitrary scanning units
300
200
100
0
Time 0 30 60 120 min
E. 12 h food intake
Food intake (g)
20
10
0
S
Insulin
TNF-α

Figure 5

TNF-α acts in the hypothalamus, inhibiting food intake and increasing the respiratory quotient without affecting early elements of the insulin signaling pathway

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Abstract

Acting in the hypothalamus, tumor necrosis factor-α (TNF-α) produces a potent anorexigenic effect. However, the molecular mechanisms involved in this phenomenon are poorly characterized. In this study, we used rats submitted to intracerebroventricular cannulation to investigate the capacity of TNF-α to activate signal transduction through elements of the pathway employed by the potent anorexigenic hormone insulin. High dose TNF-α promotes a reduction of 20 % in 12 h food intake, which is an inhibitory effect that is marginally inferior to that produced by insulin. In addition, TNF-α increases body temperature and respiratory quotient, effects not reproduced by insulin. TNF-α is also capable of activating canonical pro-inflammatory signal, in hypothalamus, inducing JNK, p38, and NFκB, which results in the transcription of early responsive genes and induction of proteins of the SOCS family. Finally, TNF-α does not activate signal transduction through early and intermediary elements of the insulin signaling pathway such as IRS-2 and Akt, however, TNF-α, even at low doses is capable of activating late elements of the insulin signaling pathway such as ERK and FOXO1. In conclusion, TNF-α exerts potent anorexigenic and pro-thermogenic effects in the hypothalamus through mechanisms independent of the activation of proteins that participate in the early and intermediary steps of the insulin signaling pathway. ERK and FOXO1, which act as late signal transducers for several signaling pathways, including insulin, are activated by TNF-α in the hypothalamus and may participate in the anorexigenic/pro-thermogenic effects of TNF-α in this organ.

Introduction

The cytokine TNF-α is one of the mediators of the anorexigenic and pro-catabolic events that lead to cachexia in cancer and severe infectious/inflammatory diseases [1, 2]. Much of the effects of TNF-α are dependent on its actions in the central nervous system (CNS), particularly in the hypothalamus [3, 4]. Interestingly, recent studies have implicated hypothalamic pro-inflammatory signaling in the development of resistance to the signals generated by anorexigenic hormones, such as insulin and leptin [5-7]. The impaired actions of these hormones in hypothalamus lead to a defective control of feeding and thermogenesis and, ultimately, may precipitate body weight gain and obesity [5].

In this context, it seems that pro-inflammatory signaling in hypothalamus, particularly promoted by TNF-α, may play a dual role in the control of feeding and thermogenesis, in some cases promoting catabolism and in other cases precipitating obesity [1, 5].

In peripheral tissues TNF-α impairs insulin signal transduction through the activation of serine kinases that participate in canonical pro-inflammatory signaling, such as JNK and IKK [8-10]. Once activated, the serine kinases catalyze the serine phosphorylation of key elements of the insulin signaling pathway such as the insulin receptor (IR) and proteins of the IRS family [10]. In addition TNF-α can stimulate the expression of proteins of the suppressor of cytokine signaling (SOCS) family, which can interact and hamper the proper activity of the IR and IRSs [11, 12].

Since TNF-α may, under different circumstances, mimic or antagonize the effects of insulin in the hypothalamus we decided to evaluate the effects of different doses of this cytokine on physiological and molecular events controlled by insulin.

Materials and methods

Antibodies, chemicals and buffers

Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA, USA). HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, bovine serum albumin

(fraction V), and angiotensin II were from Sigma (St. Louis, MO, USA). Protein A-Sepharose 6MB was from Pharmacia (Uppsala, Sweden), ^{125}I-protein A was from ICN Biomedicals (Costa Mesa, CA, USA), and nitrocellulose paper (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Sodium thiopental (Amytal) and human recombinant insulin (Humulin R) were from Lilly (Indianapolis, IN, USA). Mouse recombinant TNF-α was from Calbiochem (Darmstadt, Germany). Anti-phospho-JNK (mouse monoclonal, recognizing Thr 183 and Tyr 185, sc-6254,) c-Fos (rabbit polyclonal, sc-7202), c-Jun (rabbit polyclonal, sc-1694), phospho-ERK (mouse monoclonal, recognizing Tyr 204, sc-7383), phospho-Tyr (mouse monoclonal, sc-508), IRS-2 (rabbit plyclonal, sc-8299), IKKβ (mouse monoclonal, sc-8014), p65-NFκB (rabbit polyclonal, sc372), phospho-IκBα (rabbit polyclonal, recognizing Ser 32, sc-7977-R), phospho-p38 (mouse monoclonal, recognizing Tyr 182, sc-79773), SOCS-3 (rabbit polyclonal, sc-9023), and SOCS-2 (goat polyclonal, sc-7008) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phospho-Akt (rabbit polyclonal, recognizing Ser 473, #05-736) was purchased from Upstate Biotechnology (Charlottesville, VA, USA) and phospho-FKHR (FOXO1-rabbit polyclonal, recognizing Ser 256, #9461) was purchased from Cell Signaling Technology (Danvers, MA, USA).

Experimental animals

Eight-week old male Wistar rats (*Rattus norvegicus*) were from the University of Campinas Central Animal Breeding Center. The rats were allowed access to standard rodent chow and water *ad libitum*. All experiments were conducted in accord with the principles and procedures described by the NIH Guidelines for the Care and Use of Experimental Animals and were approved by the State University of Campinas Ethical Committee. In all experiments, the rats were submitted to intracerebroventricular (ICV) cannulation and then treated with TNF-α or insulin, according to the protocols described below.

ICV cannulation

All rats were stereotaxically instrumented using a Stoelning stereotaxic apparatus, according to a method previously described [13]. Cannula efficiency was tested one week after cannulation by the evaluation of the drinking response elicited by ICV angiotensin II [14]. Stereotaxic coordinates were: antero-posterior 0.2 mm/ lateral 1.5 mm/ depth 4.0 mm.

Protocol for food ingestion determination

ICV cannulated rats were food deprived for 6 h (from 12 to 18 h) and at 18 h were ICV treated with insulin (2.0 μl, 10^{-6} M), TNF-α (2.0 μl, 10^{-8} or 10^{-12} M) or saline (2.0 μl). Food ingestion was determined over the next 12 h.

Metabolic measurements

Rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured by open-flow respirometry, with a set-up modified from a previously published method [15]. After 14 h of fasting, rats were placed individually into polyvinyl chloride respirometric chambers, which were then installed into a temperature-controlled cabinet (± 0,1C). Air was drawn over ascarite and drierite scrubbers to remove CO_2 and H_2O to a manifold, which divided the flow into four airstreams. Each stream was regulated at 1500-2500 ml/ min, depending on the experimental temperature. The constancy of the flow was periodically checked with a Cole Palmer (Vernon Hills, IL) flow meter, and less then 1% drift during the experiments was detected. Each stream fed the inlet port of each of the four respirometric chambers; one of each contained no experimental animal and was used as a baseline reference. Air leaving each respirometric chamber was directed into a computer-controlled multiplexer, where four solenoide valves selected which flow would be directed to a computer-controlled mass-flow meter (model 840; Sierra Instruments, Inc, Monterey, CA). The mass flow meter was adjusted to deliver a constant flow of

300 ml/min of air, sequentially through water scrubbers, to the measurement cell of a CA-2A CO_2 analyzer (Sable Systems International, Las Vegas, NV), again through water and gas scrubbers, and finally to the measurement cell of a FC-1B O_2 analyzer (Sable). Each chamber with a rat was sampled for 20 min. Thus, a complete recording set lasted for 80 min, and this sequence was repeated five times during the evaluation period. Experiments started at 10:00 h and finished at 18:00 h. VO_2 and VCO_2 were calculated by the Datacan program, based in the equations of Whiters [16] and were expressed as milliliters per hour per gram. Animals were weighed immediately before and after the metabolic measurements, and the average of these two measurements was used as the body mass value for these calculations. The respiration quotient (RQ) was calculated as: VCO_2 / VO_2.

Tissue extraction, immunoblotting and immunoprecipitation

ICV cannulated rats were anesthetized and acutely treated with saline (2.0 μl), insulin (2.0 μl, 10^{-6} M) or TNF-α (in most experiments the doses of TNF-α were 10^{-8} and 10^{-12} M, 2.0 μl, except in dose response experiments when the concentrations ranged from 10^{-12} to 10^{-6} M). After 5 - 180 min, the hypothalami were obtained and immediately homogenized in solubilization buffer at 4ºC [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin/ml] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Insoluble material was removed by centrifugation for 20 min at 9,000 x *g* in a 70.Ti rotor (Beckman, Fullerton, CA, USA) at 4ºC. The protein concentration of the supernatants was determined by the Bradford dye binding method. For immunoprecipitation followed by immunoblot, aliquots of the resulting supernatants containing 2.0 mg of total protein were used for immunoprecipitation with antibodies against c-Fos, p65-NFκB or IRS-2 at 4ºC overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes and blotting with anti-c-Jun, IKKβ or phospho-Tyr. In direct immunoblot experiments, 0.2 mg of protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-phospho-JNK, phospho-p38, phospho-IκBα, c-Fos, c-Jun, phospho-Akt, phospho-ERK, phospho-FKHR (FOXO1) antibodies, as described [17].

Statistical analysis

Specific protein bands present on the blots were quantified by densitometry. Mean values ± SEM obtained from densitometric scans and from the other experiments were compared utilizing Student's *t*-test for paired samples or by repeat-measure analysis of variance (one-way or two-way ANOVA) followed by *post hoc* analysis of significance (Bonferroni test) when appropriate. A $p<0.05$ was accepted as statistically significant.

Results

TNF-α and the control of food intake

To evaluate the effect of different doses of TNF-α on feeding, ICV cannulated rats were treated with saline, insulin, low dose TNF-α (10^{-12} M), or high dose TNF-α (10^{-8} M). Spontaneous food intake was determined over the next 12 h. As shown in Figure 1, insulin promoted a 45 % reduction of food intake. High dose TNF-α produced a 20 % food intake, while low dose TNF-α was ineffective in controlling feeding.

TNF-α and the control of respirometric parameters

As shown in previous studies [18], a single ICV dose of insulin does not produce significant changes in body temperature, consumption of O_2/production of CO_2 and respiratory quotient (Fig. 2). TNF-α at a low dose, was also unable to modify body temperature and the respirometric parameters analyzed (Fig. 2). However, at a high dose, TNF-α significantly increased body temperature and the respiratory quotient while reducing O_2 consumption (Fig. 2).

Activation of pro-inflammatory signaling by TNF-α in hypothalamus

No previous study has investigated the capacity of TNF-α to activate pro-inflammatory signal transduction in the hypothalamus of rats. Therefore, we started by determining the time-course and dose-response effects of ICV injected TNF-α upon JNK,

p38 and NFκB signaling. As shown in Figure 3A, TNF-α induces the phosphorylation-activation of JNK, starting at a dose of 10^{-10} M and reaching a peak at 10^{-8} M. Using the dose of 10^{-8} M we observed that TNF-α action upon JNK started at 10 min and lasted for at least 20 min (Fig 3B). In addition, TNF-α induced the phosphorylation-activation of p38 at the dose of 10^{-8} M, but not at the dose of 10^{-12} M (Fig. 3C). Similarly, at the dose of 10^{8} M TNF-α induced the association of IKK with p65-NFκB (Fig. 3D) and the phosphorylation activation of IκB (Fig. 3E). Interestingly, both the low and the high doses of TNF-α were capable of inducing the expression of the early responsive proteins, c-Jun (Fig. 3F) and c-Fos (Fig. 3G); however, only the high dose of TNF-α promoted the expression of the cytokine responsive suppressors of signaling SOCS-2 (Fig. 3H) and SOCS-3 (Fig. 3I).

TNF-α action on elements of the insulin signaling pathway

A single ICV dose of insulin promoted a 2.5-fold increase of Tyr-phosphorylation of IRS-2 (Fig. 4A) and a 1.8-fold increase of Ser^{473}-phosphorylation of Akt (Fig. 4B). TNF-α, either at low or at high doses, was unable to activate signal transduction through IRS-2 and Akt (Fig. 4A and 4B). Downstream, insulin promoted a 1.5-fold increase in phosphorylation of ERK (Fig. 4C), and a 2.0-fold increase phosphorylation of FOXO1 (Fig. 4D). In addition, both low and high doses of TNF-α promoted significant increases in phosphorylation of ERK and FOXO1 (Fig. 4C and 4D). The magnitudes of the effects of TNF-α upon ERK and FOXO1 were similar to that produced by insulin.

Discussion

The prime objective of this study was to evaluate the effect of TNF-α upon physiological and molecular events controlled by insulin in the hypothalamus. Classically, TNF-α is regarded as a potent anorexigenic and pro-catabolic cytokine [1,19].

However, recent studies have suggested that, under certain circumstances, increased hypothalamic levels of TNF-α can coincide with increased feeding and obesity [5]. Apparently, in these conditions, TNF-α impairs anorexigenic insulin signal transduction by the activation of serine kinases that catalyze the serine phosphorylation of the IR and IRS proteins, therefore, hampering their appropriate responses to insulin [5]. Since the effects of pro-inflammatory signaling upon insulin action were observed in an animal model of diet-induced obesity, we suspect that it results from the chronic stimulation of signal transduction by TNF-α, and possibly by other cytokines [5, 7, 20]. Conversely, when a single, high dose of TNF-α is injected in the CNS it leads to a remarkable inhibition of feeding [21]. The mechanisms behind this acute effect are poorly understood but may involve the control of hypothalamic neurotransmitter expression [3, 22], *in situ* stimulation of the expression of other cytokines, particularly IL-1β [4, 23], and the activation of anorexigenic leptin-like signal transduction in the hypothalamus [24]. Since insulin acts in conjunction with leptin to deliver the most robust anorexigenic signals to the hypothalamus, we hypothesized that TNF-α could act upon elements of the insulin signaling pathway to exert, at least part of its anorexigenic functions.

Initially, we evaluated the acute effect of a high and a low dose of TNF-α upon the control of feeding. As expected, the effect of insulin was remarkable, leading to an almost 50 % reduction in food intake. TNF-α at a high dose, promoted a more discrete effect, reducing food intake by 25 %, while at a low dose the cytokine was devoid of function. Next, we determined the effect of TNF-α upon body temperature and consumption of O_2/production of CO_2. Very few studies have previously looked upon the acute, ICV effect of insulin on respirometric parameters [18]. According to these studies and to the data shown here, insulin alone exerts no acute effect on body temperature, consumption of O_2 and production of CO_2. Based on recent studies, we can assume that insulin, acting alone, preferentially activates signal transduction through phosphatidylinositol 3-kinase/Akt [25, 26]. Once activated, this pathway controls the release of anorexigenic neurotransmitter in neuron terminals. Conversely, when acting in concert with leptin, insulin induces the activation of STAT-3 signaling, which drives the signal towards the control of anorexigenic and pro-thermogenic gene expression [26].

In the respirometric assay we observed that the high dose of TNF-α promoted a significant increase of body temperature, which was accompanied by a reduction of O_2 consumption and by an increase of respiratory quotient. These results are in accordance with the well-known catabolic effects of TNF-α and reinforce the idea that much of this effect is dependent on hypothalamic signaling and does not overlap with insulin action. Once more, the low dose of TNF-α exerted no effect on these parameters.

In peripheral tissues, TNF-α activates signal transduction through several intermediaries; JNK, p38 and NF B are amongst the most important mediators of TNF-α action [8, 10, 27]. In this study, we observed that a high dose of TNF-α was capable of activating signal transduction through JNK, p38 and NF B. Moreover, the same dose of TNF-α was also capable of inducing the expression of c-Jun, c-Fos, SOCS-2 and SOCS-3. Therefore, we can admit that the hypothalamus of naive rats is appropriately equipped with the molecular apparatus required for TNF-α action. Although we have not evaluated the expression of the different forms of TNF-α receptors, it has been previously shown that TNFR1 predominates over TNFR2 in this site [28] and, thus, we suspect that it is responsible for transducing most of the signaling events detected in this study.

Finally, with regard to the capacity of TNF-α to stimulate elements of the insulin signaling pathway, we observed that neither the high dose nor the low dose of the cytokine produced significant effects on IRS-2 and Akt. However, when looking at distal elements of the pathway both doses of TNF-α produced effects that are similar to the effects produced by insulin. Interestingly, although the low dose of TNF-α was unable to transduce the signal towards the intermediaries herein analyzed, both in the pro-inflammatory and in the metabolic pathways, it promoted the expression of c-Jun and c-Fos and the activation of ERK and FOXO-1. Therefore, we suspect that even at very low levels, TNF-α can stimulate other signaling pathways not explored in this study, and this may have implications in situations that couple with chronic, low production of pro-inflammatory cytokines, such as, for example, obesity.

In conclusion, high dose, acutely injected TNF-α acts in the hypothalamus, reducing food intake and shifting energetic substrate usage to favor catabolism.

These effects are coupled with canonical pro-inflammatory signaling and are independent of the engagement of elements that participate in early insulin signal transduction. Low and high doses of TNF-α can activate gene expression in the hypothalamus and also activate promiscuous late elements of the insulin signaling pathway. The acute effects of TNF-α upon feeding are, therefore, independent of the activation of classical insulin signaling.

Acknowledgements

This study received grants from Fundação de Amparo à Pesquisa do Estado de São Paulo, from Conselho Nacional de Desenvolvimento Científico e Tecnológico and from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior.
We thank Dr. N. Conran for English grammar editing.

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Legends for the figures

Figure 1- Twelve-hour food intake determination was performed in ICV cannulated rats. For this, the animals were food deprived from 12 to 18 h and then treated with 2.0 µl saline (ct), insulin (10^{-6} M) (i), or TNF-α [10^{-12} M (t^{-12}) or 10^{-8} M (t^{-8})]. Food was reintroduced and spontaneous ingestion was determined over the next 12 h. In all experiments, n=4; *p<0.05 *vs.* saline treated (ct); §p<0.05 *vs.* insulin treated (i). Results are presented as mean ± SEM.

Figure 2- ICV cannulated rats were treated with 2.0 µl saline (ct), insulin (10^{-6} M) (i), or TNF-α [10^{-12} M (t^{-12}) or 10^{-8} M (t^{-8})] and placed into polyvinyl chloride respirometric chambers for determination of respirometric parameters, as described in the Materials and methods section. Body temperature (A), respiratory quotient (RQ) (B), consumption of O_2 (vO_2) (C) and production of CO_2 (vCO_2) (D) were obtained as described. In all experiments, n=4; *p<0.05 *vs.* saline treated (ct). Results are presented as mean ± SEM.

Figure 3- Anesthetized rats were ICV treated with 2.0 µl, TNF-α at 10^{-8} M (B) or at the concentrations depicted in the figures. After the time points, as depicted in the figure (B) or after 10 min (A, C-E), 30 min (H and I) or 180 min (F and G), hypothalami were obtained for protein extract preparation. For direct immunoblot analysis (A-C, E-I), samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific antibodies to phospho-JNK (p-JNK) (A, B); phospho-p38 (p-p38) (C); phospho-IκB (p-IκB)(E), c-Jun (F), c-Fos (G), SOCS-2 (H) and SOCS-3 (I). For immunoprecipitation followed by immunoblot, samples containing 2.0 mg total protein were submitted to immunoprecipitation (IP) with antibodies to c-p65-NFκB (p65) (D); immunoprecipitates were collected with Protein A-Sepharose and separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with antibodies to IKK. Specific bands were quantified by densitometric analysis. In all experiments, n=4; *p<0.05 *vs.* time 0 (B) or *vs.* concentration 0 (A, C-I). Results are presented as means ± SEM.

Figure 4- Anesthetized rats were ICV treated with 2.0 μl saline (ct), insulin (10^{-6} M) (i), or TNF-α [10^{-12} M (t^{-12}) or 10^{-8} M (t^{-8})]. After 10 min (A-C) or after 15 min (D) hypothalami were obtained for protein extract preparation. For direct immunoblot analysis (B-D), samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific antibodies to phospho-Akt (p-Akt) (B); phospho-ERK (p-ERK) (C); or phospho-FOXO1 (p-FOXO1) (D). For immunoprecipitation followed by immunoblot, samples containing 2.0 mg total protein were submitted to immunoprecipitation (IP) with antibodies to IRS-2 (A); immunoprecipitates were collected with Protein A-Sepharose and separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with antibodies to phospho-Tyr (pY). Specific bands were quantified by densitometric analysis. In all experiments, n=4; *$p<0.05$ *vs.* ct. Results are presented as mean ± SEM.

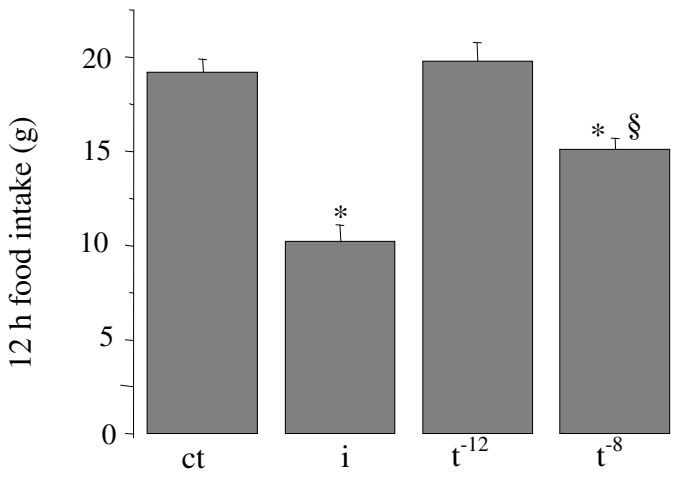

Figure 1

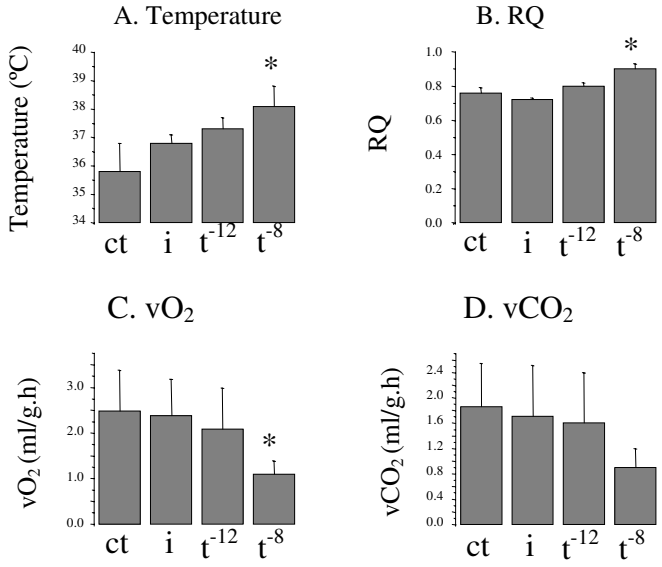

Figure 2

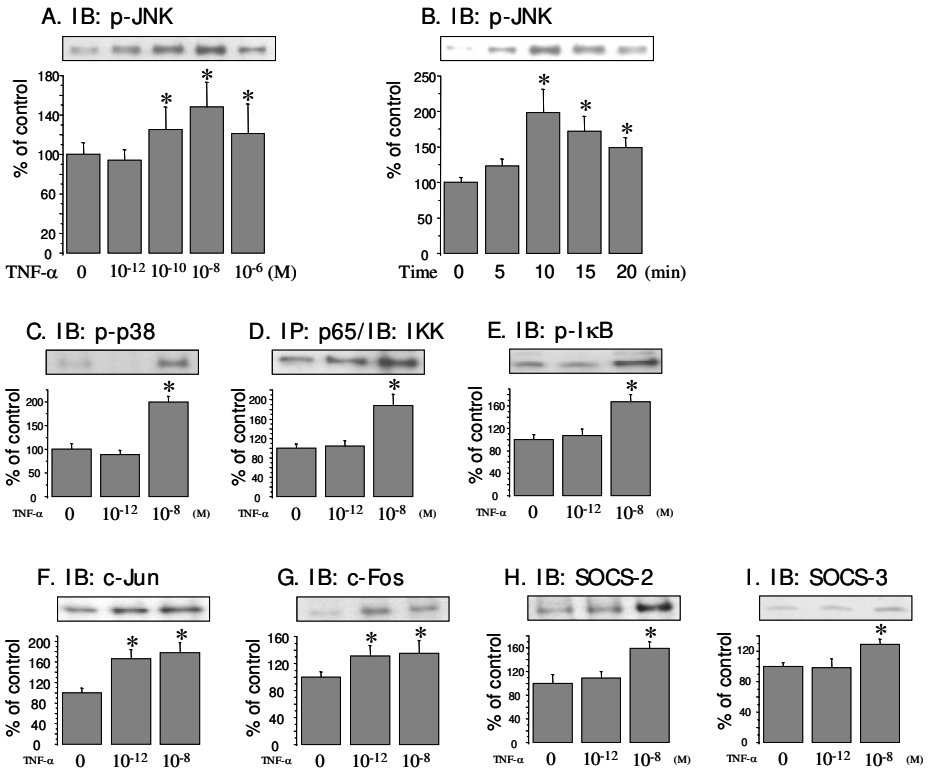

Figure 3

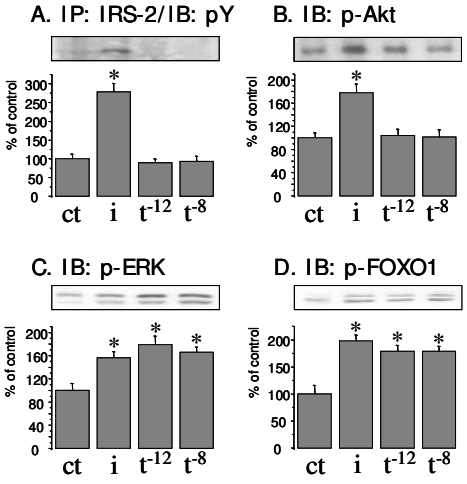

Figure 4

4- DISCUSSÃO GERAL

As vias de sinalização do TNF-α vêm sendo estudadas por vários grupos em células do sistema imune e em diversos outros tecidos. No entanto, no sistema nervoso central e, mais especificamente, no hipotálamo, pouco se conhece a respeito da transdução do sinal desta citocina.

Nesse estudo nós avaliamos a transdução do sinal da via pró-inflamatória e da via da insulina pelo TNF-α e a ativação de genes responsivos a ele no hipotálamo de ratos, além de eventos fisiológicos tais como a ingestão alimentar e o consumo de O_2/produção de CO_2.

Na primeira parte do estudo, nós avaliamos a capacidade de uma dose aguda de TNF-α ativar proteínas envolvidas na resposta pró-inflamatória, como JNK, p38, NFκB, IKK e SOCS. Esses eventos foram acompanhados da indução do complexo AP-1 e da expressão de c-Fos e c-Jun. Nós acreditamos que esses efeitos são especificamente dependentes do TNF-α, visto que o pré-tratamento dos animais com o anticorpo monoclonal anti-TNF-α, Infliximab, preveniu completamente a ativação da transdução do sinal. Além disso, observamos que o TNF-α foi capaz de induzir ativação de transcrição gênica por meio de, pelo menos, um promotor, o promotor de *c-jun,* ratificando a integridade de toda a via de sinalização desde a membrana celular até o núcleo.

Nessa parte do estudo nós observamos que uma dose alta de TNF-α(10^{-8}M) é capaz de ativar JNK, p38 e NFκB e induzir a expressão de SOCS-2 e SOCS-3 no hipotálamo. Ambas as doses, alta (10^{-8}M) e baixa (10^{-12}M), foram capazes de induzir a expressão de c-Jun e c-Fos. Portanto, nós podemos admitir que o hipotálamo de ratos é apropriadamente equipado com todo o aparato molecular necessário para a ação do TNF-α neste sítio anatômico. Apesar de não termos avaliado a expressão das diferentes formas do receptor de TNF-α, foi previamente descrito que tanto TNFR-1 como TNFR-2 estão presentes nessa região, com predomínio do primeiro sobre o segundo (Nadeau and Rivest, 1999). Desta forma, nós suspeitamos que aquele seja o principal responsável pela transdução dos sinais detectados nesse estudo.

Posteriormente, nós avaliamos os efeitos do TNF-α sobre a expressão de duas citocinas pró-inflamatórias (IL-1β e IL-6) e uma citocina anti-inflamatória (IL-10) e duas proteínas responsivas a citocinas, SOCS-2 e SOCS-3. As três citocinas foram rapidamente

induzidas em resposta ao TNF-α, tendo início aos 15 minutos e se prorrogando por até 3 horas. Nos nossos experimentos, a indução de IL1-β, IL-6 e IL-10 pelo TNF-α foi rápida, sugerindo ser este um evento primário, não necessitando de intermediários. Em relação a SOCS-2 e SOCS-3, inicialmente acreditava-se que a expressão dessas proteínas era exclusivamente controlada por receptores da família de receptores de citocinas do tipo I (Auernhammer and Melmed, 2001). Mais recentemente, demonstrou-se que a SOCS-3 pode ser induzida também por citocinas e hormônios que agem através de outras classes de receptores, incluindo TNF-α (Bjorbaek et al., 1998; Calegari et al., 2003; Emanuelli et al., 2001; Rui et al., 2002; Torsoni et al., 2004). A capacidade do TNF-α em induzir SOCS-3 foi demonstrada em tecido adiposo (Emanuelli et al., 2001), fígado (Campbell et al., 2001; Sass et al., 2005) e em células do sistema imune (Bode et al., 2003). No entanto, nenhum estudo havia demonstrado a capacidade do TNF-α induzir SOCS-3 (ou outros membros da família SOCS) em tecidos neurais. Devido ao longo tempo necessário para o TNF-α induzir SOCS-3 no nosso estudo, nós não podemos afirmar com certeza se este é um evento primário ou se depende da expressão de intermediários.

Ainda na primeira parte do trabalho, nós avaliamos a capacidade do TNF-α em modular a expressão de neurotransmissores no hipotálamo. Usando RT-PCR e imunoblot, nós observamos que ambos, peptídeos orexigênicos e anorexigênicos, foram significantemente e rapidamente induzidos pelo tratamento com a dose mais alta (10^{-8}M) de TNF-α, sendo que os níveis de peptídeos anorexigênicos mostraram-se consideravelmente mais elevados. Portanto, nós acreditamos que a maior expressão de neuropeptídeos anorexigênicos desempenha importante papel na ação do TNF-α no hipotálamo, que foi claramente anorexigênico nessa dose.

Até aqui pudemos demonstrar que o TNF-α ativa uma cascata de transdução de sinal no hipotálamo de ratos. Esses eventos induzem a expressão de citocinas e outras proteínas relacionadas com a sinalização inflamatória que podem ter implicações na resposta local a estímulos pró-inflamatórios. O TNF-α, por si só, na dose mais alta (10^{-8}M) modula a expressão de neurotransmissores envolvidos no controle da homeostase energética, favorecendo o gasto energético.

Classicamente, o TNF-α é uma potente citocina com papel anorexigênico e pró-catabólico (Laviano et al., 2003; Matthys and Billiau, 1997). No entanto, estudos recentes têm sugerido que, sob certas circunstâncias, o aumento dos níveis hipotalâmicos de TNF-α coincidem com um aumento na ingestão alimentar e obesidade (De Souza et al., 2005). Aparentemente nessas condições, o TNF-α interfere negativamente na transdução do sinal anorexigênico da insulina por meio da ativação de serina quinases que catalisam a fosforilação em serina do IR e IRSs, impedindo assim uma resposta apropriada frente ao estímulo com insulina (De Souza et al., 2005). De acordo com os efeitos da sinalização pró-inflamatória sobre a ação da insulina observados num modelo animal de obesidade induzida por dieta, nós suspeitamos que isso pudesse resultar de uma estimulação crônica via TNF-α e, possivelmente, outras citocinas (De Souza et al., 2005; Flier, 2004; Howard et al., 2004).

Contrariamente, como visto no nosso trabalho, quando uma única dose alta de TNF-α é injetada no sistema nervoso central ocorre uma acentuada inibição da ingestão (Plata-Salaman et al., 1996). Os mecanismos por trás desse efeito agudo são pouco conhecidos, mas envolvem o controle da expressão de neurotransmissores hipotalâmicos (Bernardini et al., 1990; Wisse et al., 2003) a estimulação *in situ* da expressão de outras citocinas, particularmente IL-1β (Bristulf and Bartfai, 1995; Zhai et al., 1997) e da ativação de vias anorexigênicas dependentes da leptina no hipotálamo (Rizk et al., 2001). Pelo fato da insulina agir em conjunto com a leptina para promover os sinais anorexigênicos para o hipotálamo, nós imaginamos que o TNF-α pudesse agir sobre elementos da via de sinalização da insulina para exercer, em parte, suas funções anorexigênicas.

Portanto, numa segunda etapa do trabalho nós avaliamos o efeito agudo de uma dose alta (10^{-8}M) e de uma dose baixa (10^{-12}M) sobre a ingestão alimentar. Como esperado, a insulina promoveu uma redução de aproximadamente 50% na ingestão, enquanto a dose mais alta de TNF-α promoveu um efeito mais discreto, reduzindo a ingestão em aproximadamente 25%. A dose mais baixa não produziu efeitos sobre a ingestão. Posteriormente, nós determinamos o efeito do TNF-α sobre a temperatura corpórea e o consumo de O_2/produção de CO_2. Poucos estudos relataram o efeito agudo da insulina

sobre parâmetros respirométricos (Menendez and Atrens, 1991). De acordo com esses estudos e com os dados mostrados aqui, a insulina sozinha não exerce efeito sobre a temperatura, consumo de O_2 e produção de CO_2. Baseado em estudos recentes, a insulina, agindo sozinha, ativa preferencialmente a transdução de sinal através da via PI3K/Akt (Carvalheira et al., 2001; Xu et al., 2005). Uma vez ativada essa via controla a liberação de neurotransmissores anorexigênicos em terminais nervosos.

De acordo com os dados de respirometria, nós observamos que a dose mais alta de TNF-α promoveu um significante aumento na temperatura corpórea, que foi acompanhado de uma redução no consumo de O_2 e um aumento do quociente respiratório. Esses resultados estão de acordo com o que já se sabe a respeito dos efeitos catabólicos do TNF-α e reforçam que muitos desses efeitos são dependentes da sinalização dessa citocina no hipotálamo e não apenas da insulina. A dose mais baixa não modulou esses parâmetros.

Finalmente, levando-se em conta a capacidade do TNF-α em modular negativamente elementos da via de sinalização da insulina, nós observamos que tanto a dose alta quanto a dose baixa da citocina foram incapazes de ativar elementos clássicos da via da insulina, como, IRS-2 e Akt. No entanto, quando avaliamos elementos distais da via, ambas as doses de TNF-α produziram efeitos que são similares aos produzidos pela insulina. Apesar da dose baixa de TNF-α ser incapaz de ativar elementos proximais e intermediários que foram analisados tanto na via pró-inflamatória quanto na via metabólica, ela induz a expressão de c-Jun e c-Fos e a ativação de ERK e FOXO-1. Portanto, nós suspeitamos que, mesmo em concentrações baixas, o TNF-α pode estimular outras vias de sinalização não exploradas nesse estudo, e pode estar envolvido em situações que se associam com a produção crônica e discreta de citocinas pró-inflamatórias, como por exemplo, na obesidade.

Concluindo, uma dose alta, infundida de forma aguda, age no hipotálamo aumentando a expressão de neuropeptídeos anorexigêncios, reduzindo a fome e promovendo alteração no substrato energético utilizado favorecendo o catabolismo. Esses efeitos estão em concordância com a sinalização pró-inflamatória e são independentes do engajamento de elementos que participam dos mecanismos iniciais de

transdução do sinal da insulina. Doses baixas e altas de TNF-α podem ativar a expressão gênica no hipotálamo e também ativar elementos distais da via de sinalização da insulina. Os efeitos agudos do TNF-α sobre a ingestão alimentar são, portanto, independentes da ativação de elementos clássicos da via da insulina.

5- CONCLUSÃO GERAL

Estudos recentes têm sugerido que a resistência hipotalâmica à ação dos hormônios anorexigênicos, leptina e insulina, desempenha um papel central na gênese da obesidade e deve representar uma conexão importante entre essa doença e o diabetes mellitus tipo 2. O consumo de dieta rica em lipídeos parece ser um fator ambiental capaz de exercer um efeito relevante na gênese da resistência à ação desses hormônios por induzir a expressão de proteínas da família SOCS que inibem a sinalização da insulina e da leptina (Howard et al., 2004) e por promover a ativação de vias pró-inflamatórias no hipotálamo ativando a serina-quinase JNK e induzindo a fosforilação em serina do IR e do IRS-2 (De Souza et al., 2005). Um dos mecanismos através dos quais o consumo de dieta rica em lipídeos pode promover a ativação da sinalização pró-inflamatória é a indução da expressão de citocinas tais como TNF-α, IL-1β e IL-6 no hipotálamo.

Neste estudo avaliaram-se os efeitos da infusão de TNF-α no hipotálamo de ratos. Observou-se que, na dose mais alta utilizada, o TNF-α exerce um efeito importante sobre a ativação da sinalização celular pró-inflamatória, sobre a produção de neuropeptídeos anorexigênicos e sobre o controle da ingestão alimentar e consumo de O_2. Esses efeitos são, no entanto, independentes da ação do TNF-α sobre a via de sinalização da insulina nesse tecido.

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7- APÊNDICES

Materiais e métodos

Animais experimentais

Foram utilizados ratos (*Rattus norvergicus*) machos, da linhagem Wistar entre quatro e dez semanas de idade, provenientes do Biotério Central da UNICAMP (CEMIB), os quais foram alimentados com ração comercial para roedores (Nuvilab CR-1) da Nuvital, oferecida *ad libitum*, assim como água. Os animais foram mantidos em gaiolas coletivas (cinco por gaiola) sob condições padronizadas de iluminação (ciclo claro/escuro de 12 horas) e temperatura de 22 ± 2° C.

Os animais foram randomicamente divididos em seis grupos: tratados com salina (C); tratados com insulina 10^{-6} M (I); tratados com TNF-α 10^{-12} M (T12); tratados com TNF-α10^{-10} M (T10); tratados com TNF-α10^{-8} M (T8); tratados com TNF-α 10^{-6} M (T6).

Cirurgia Estereotáxica

Ao atingirem 8 semanas de idade os ratos foram submetidos à canulação do ventrículo lateral. Os animais (250 - 300g) foram previamente anestesiados por via intraperitonial com uma mistura de 1:1 (0,8 ml) de cloridrato de cetamina (50 mg/ml) e diazepan (5,0 mg/ml). Depois de testados os reflexos corneano e pedioso os animais foram posicionados no aparelho de estereotaxia para implantação de uma cânula no ventrículo lateral do hipotálamo, seguindo as coordenadas do Atlas Paxinos-Watson (Paxinos et al., 1980) que variam de acordo com o peso do animal. Em ratos de aproximadamente 300 g as coordenadas utilizadas foram AP: 0,2 mm; Lateral: 1,5 mm; Profundidade: 4 mm.

Infusão intracerebroventricular

Após o período de uma semana de recuperação da cirurgia estereotáxica, os animais foram submetidos a um teste de resposta de ingestão hídrica subseqüente ao tratamento com angiotensina II (2,0 μl de solução 10^{-6} M) para avaliação da adequação da posição da cânula. Ratos com resposta positiva à angiotensina II foram selecionados e

utilizados nos experimentos. Animais de cada situação experimental receberam 2,0 μl ICV de salina, insulina ou TNF-α nas concentrações descritas acima.

Para a infusão intracerebroventricular das drogas ou salina foi utilizada uma seringa Hamilton (50 μl) acoplada, por meio de uma cânula plástica, a uma agulha 30G, de modo que esta ultrapassasse o comprimento da cânula metálica (0,1 - 0,2 mm) garantindo a entrada do líquido no ventrículo lateral.

Avaliação da ingestão alimentar

Os animais foram colocados individualmente em gaiolas metabólicas e mantidos em jejum por 6 horas. Às 18 h os ratos foram submetidos aos diferentes tratamentos por via ICV e a ingestão de 12 horas foi determinada pela aferição da diferença de peso entre o alimento oferecido e o alimento restante na gaiola.

Dissecção do hipotálamo

Os animais foram anestesiados por meio da administração intraperitonial de tiopental sódico (0,6 ml; 15 mg/kg) e a perda dos reflexos pedioso e corneano foi utilizada como controle da anestesia. Após os diferentes tratamentos, o crânio foi aberto, o hipotálamo retirado e em seguida homogeneizado em aproximadamente 10 volumes de tampão de solubilização (1% Triton X-100; 100 mM Tris pH 7,4; contendo 100 mM de pirofosfato de sódio; 100 mM de fluoreto de sódio; 10 mM de vanadato de sódio; 2 mM PMSF e 0,1 mg de aprotinina/mL) a 4° em "Politron PTA 20S generator" (Brinkmann Instruments mode PT 10/35) com velocidade máxima por 30 segundos. O homogeneizado foi então centrifugado a 11.000 rpm por 30 minutos para remoção de material insolúvel. No sobrenadante foi determinada a concentração de proteínas utilizando-se o método de Bradford (Bradford, 1976).

Imunoprecipitação

Nos experimentos nos quais se desejava verificar a fosforilação de proteínas, das quais não havia anticorpos específicos fosforilados, ou, quando se desejava verificar associação entre proteínas, foi empregado o método de imunoprecipitação. As amostras solubilizadas foram centrifugadas a 11000 rpm por 30 min a 4°C e o sobrenadante foi retirado para imunoprecipitação. Os volumes das amostras foram normalizados por concentração protéica (1,0 mg de proteína total por amostra). As amostras foram incubadas por 12 a 14 horas, a 4°C. Após incubação, os imunocomplexos foram recuperados com Proteína A Sepharose 6MB por 2 horas a 4°C e decantados por centrifugação por 15 minutos a 4°C/11.000 rpm. O precipitado foi lavado três vezes, em intervalos de 5 minutos, com tampão de lavagem (2,0 mM ortovanadato de sódio, 100 mM Tris-Hcl, 10 mM EDTA, 0,5% Triton X-100). O sobrenadante foi descartado retendo-se apenas as proteínas precipitadas (imunocomplexos) (Velloso et al., 1996). Os imunocomplexos foram ressuspensos em 25 μl de tampão de Laemmli, contendo 100 mmol/l de DTT.

Imunoblot

Após rápida fervura (5 minutos) as amostras, tanto de imunoprecipitado quanto de extrato total (contendo 0,2 mg de proteína total), foram aplicadas em gel de poliacrilamida para separação por eletroforese (SDS-PAGE). As proteínas separadas por SDS-PAGE foram transferidas para membrana de nitrocelulose em aparelho de transferência da BIO-RAD durante 120 min a 80 Volts, em gelo, e banhadas com tampão de transferência. As membranas de nitrocelulose foram incubadas por 12 a 14 horas com anticorpo específico. A ligação do anticorpo a proteínas não específicas foi minimizada pela pré-incubação das membranas de nitrocelulose com tampão de bloqueio (5% de leite em pó desnatado; 10 mmol/l de Tris, 150 mmol/l de NaCl, 0,02% de Tween 20) por 1,5 hora. A detecção do complexo antígeno-anticorpo fixo à membrana de nitrocelulose foi obtida por meio de tratamento com ^{125}I-proteína A em 10 ml de tampão de bloqueio por 2 horas em temperatura ambiente e exposição a filmes de RX Kodak a 80°C por

36 - 72 horas. Após a revelação das auto-radiografias as bandas obtidas foram quantificadas por meio de densitometria óptica (Araujo et al., 2005; Velloso et al., 1996).

Calorimetria indireta

Após jejum prévio de 14 horas, os animais pertencentes aos respectivos grupos descritos acima foram colocados individualmente em câmaras respiratórias de PVC, com volume de 4 litros cada, mantidos à temperatura controlada. Para análise do consumo de O_2 e produção de CO_2 o ar foi direcionado a uma coluna contendo ascarite e gel sílica, para remoção do CO_2 e da água, respectivamente, antes de chegar a um distribuidor com múltiplas saídas, o qual dividiu o fluxo total em quatro ou cinco canais, dependendo do número de animais analisados por vez. Cada canal foi regulado para um fluxo de 1.500 a 2.500 ml/min de ar, dependendo da temperatura experimental. O fluxo de ar de cada respirômetro foi periodicamente checado por um fluxímetro modelo Cole-Palmer para detecção de oscilações menores que 1% durante as medições. Cada um dos 4 ou 5 canais de fluxo alimentou um respirômetro, sendo que, um deles não continha animal e foi usado como referência basal. O fluxo de ar de cada respirômetro foi direcionado a um multiplexador, controlado por computador, o qual continha quatro ou cinco válvulas solenóides direcionadas a um fluxímetro de massa (Sierra - modelo 840) controlado por computador. O fluxímetro de massa foi ajustado para liberar um fluxo constante de 300 ml/min de ar, seguido da retirada da umidade do ar para análise do CO_2 (CA-2A, Sable System) e novamente retirada da umidade e do CO_2 do ar total e finalmente análise de O_2 (CA-2A, Sable System). Uma interface do computador com um conversor A/D (UI-2, Sable System), controlado por um software de coleção de dados (Datacan V.5, Sable System) regulou qual fluxo de ar deveria ser direcionado para o circuito de analisador de gás, controlou o fluxímetro de massa, leu e armazenou os resultados dos gases analisados. Para controlar resultados apropriados com o programa Datacan, as amostras de ar de cada respirômetro foram coletadas continuamente e sequencialmente. Amostras da câmara basal foram coletadas 10 minutos antes e depois das câmaras que continham animais e as que continham animais foram avaliadas por 20 minutos. Essa seqüência foi repetida por cinco vezes de modo que cada animal foi avaliado num total de 100 minutos.

O VO_2 e o VCO_2 foram calculados pelo programa Datacan baseados na equação de Withers (Withers, 1977) e foram expressos como $ml.h^{-1}.g^{-1}$. Os animais foram pesados imediatamente antes e após as medidas metabólicas e a média das duas pesagens foi usada como valor de massa corporal nos cálculos. O quociente respiratório (QR) foi calculado como VCO_2/VO_2.

Análise estatística

Os resultados foram expressos como média ± erro padrão da média. Quando comparados dois grupos, foi utilizado o teste t de Student para dados não pareados. Quando necessário, utilizou-se análise de variância (ANOVA), seguida de teste para comparação múltipla de médias. Foi adotado o nível de significância $p<0.05$.