

EDUARDO ROCHETE ROPELLE

**Papel da AMPK Hipotalâmica no Desenvolvimento da Anorexia
em Ratos com Tumor de Walker-256**

CAMPINAS

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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 Clínica Médica,
área de concentração em Ciências Básicas.*

Orientador: Prof. Dr José Barreto Campello Carvalheira

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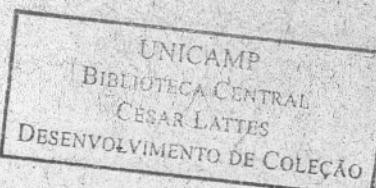


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LISTA DE ABREVEATURAS

ACC	Acetil CoA carboxilase
ACTH	Hormônio adenocorticotrófico
AG	Ácido graxo
AgRP	Proteína relacionada ao Agouti
AICAR	<i>5-amino-4 imidazolecarboxamide-riboside</i>
AMP	Adenosina monofosfato
AMPK	Proteína quinase ativada por AMP
ARC	Núcleo arqueado do hipotálamo
ATP	Adenosina trifosfato
C75	Composto 75
CART	<i>cocaine-and amphetamine-regulated transcription</i>
C/EBP	<i>CCAAT/enhancer-binding protein</i>
COBEA	Colégio brasileiro de experimentação animal
CPT-1	Carnitina palmitoil transferase 1
COX-2	Ciclo-oxigenase-2
EDTA	Ácido etilenodiaminotetracético
ELISA	<i>Enzyme Linked Immuno Sorbent Assay</i>
eNOS	Óxido nítrico endotelial
i.c.v.	Intracerebroventricular
IFN- γ	Interferon gama
iNOS	Óxido nítrico sintetase induzível
IL-1 β	Interleucina 1 beta

IL-1R1	Receptor 1 de interleucina 1
IL-6	Interleucina 6
i.p.	Intraperitoneal
ITT	Teste de tolerância à insulina
JAK	Proteína Janus quinase
Kitt	Taxa de remoção da glicose
LHA	núcleo hipotalâmico lateral
LPS	Lipopolissacarídeo
MAPK	Proteína quinase ativadora de mitose
MnSOD	Superóxido dismutase dependente do manganês
mRNA	Ácido ribonucleico (mensageiro)
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NF-κB	Fator nuclear kappa B
nNOS	Óxido nítrico neuronal
NaCl	Cloreto de sódio
Na ₄ P ₂ O ₇	Pirofosfato de sódio
NO	Óxido nítrico
NOS	Óxido nítrico sintase
NPY	Neuropeptídeo Y
<i>ob/ob</i>	Camundongo deficiente de leptina
OBR	Receptor de leptina
OBR _L	Receptor de leptina de forma longa
OBR _S	Receptor de leptina de forma curta
PI 3-quinase	fosfatidilinositol 3-quinase

POMC	Proopiomelanocortina
PVN	Núcleo paraventricular do hipotálamo
RIA	Radio-imuno ensaio
RNA	Ácido ribonucleico
RT	Transcriptase reversa
S.E.M.	Erro padrão
SNC	Sistema nervoso central
STAT-3	Transdutor de sinal e ativador de transcrição 3
TNF- α	Fator de necrose tumoral
VMH	Núcleo ventromedial do hipotálamo
Tris	Tri (hidroximetil)-aminometano
2DG	2 deoxi-D-glicose
α -MSH	Hormônio estimulador de melanócito alfa

RESUMO

A patogênese da anorexia induzida pelo câncer é multifatorial e está associada a distúrbios dos mecanismos centrais que controlam a ingestão alimentar. Todavia, os mecanismos neuroquímicos responsáveis pelo desenvolvimento da anorexia induzida pelo câncer são desconhecidos. Este presente estudo demonstra que a infusão crônica de 5-amino-4 imidazolecarboxamide-riboside (AICAR) no terceiro ventrículo hipotalâmico e a injeção intraperitoneal de 2 deoxi-D-glicose (2DG), promoveram a ativação da AMPK hipotalâmica, aumentaram a ingestão alimentar e prolongaram a sobrevida de ratos com anorexia induzida por tumor. Em paralelo, a ativação farmacológica da AMPK hipotalâmica em animais portadores de tumor, reduziu significativamente a produção hipotalâmica da óxido nítrico sintase induzível (iNOS), da Interleucina-1 β (IL-1 β) e do fator de necrose tumoral alfa (TNF- α) e modulou a expressão de neuropeptídeos hipotalâmicos (NPY e POMC) envolvidos no controle da homeostase energética. Além disso, o tratamento diário via oral com metformina, biguanida com ação anti-diabética, também aumentou a fosforilação da AMPK hipotalâmica, aumentou a ingestão alimentar e a sobrevida nos animais com anorexia induzida pelo tumor. Coletivamente, nossos achados sugerem que a ativação da AMPK hipotalâmica reverte a anorexia induzida pelo câncer através da inibição da produção de moléculas pró-inflamatórias e através do controle da expressão de neuropeptídeos no hipotálamo, refletindo em aumento da sobrevida dos ratos portadores de tumor. Assim nossos dados indicam que a ativação da AMPK representa uma atrativa oportunidade para o tratamento da anorexia induzida pelo câncer.

ABSTRACT

The pathogenesis of cancer anorexia is multifactorial and is associated with disturbances of the central physiological mechanisms controlling food intake. However the neurochemical mechanisms responsible for cancer induced-anorexia are unclear. Here, we show that chronic infusion of 5-amino-4imidazolecarboxamide-ribose (AICAR) into the third cerebral ventricle and a chronic peripheral injection of 2 deoxy-D-glucose (2DG) promotes hypothalamic AMPK activation, increased food intake and prolongs the survival of anorexic tumor bearing (TB) rats. In parallel, the pharmacological activation of hypothalamic AMPK in TB animals, markedly reduced the hypothalamic production of inducible nitric oxide synthase (iNOS), interleukin-1 β IL-1 β and tumor necrosis factor-alpha (TNF- α) and modulated the expression of hypothalamic neuropeptides (NPY and POMC) involved in the control of energy homeostasis. Furthermore, the daily oral treatment with biguanide anti diabetic drug metformin also induced AMPK phosphorylation in the central nervous system and increased food intake and life span in anorexic TB rats. Collectively, the findings of this study suggest that hypothalamic AMPK activation reverses cancer anorexia by inhibiting the production of proinflammatory molecules and controlling the neuropeptide expression in the hypothalamus, reflecting in a prolonged life span in TB rats. Thus, our data indicate that hypothalamic AMPK activation presents an attractive opportunity for the treatment of cancer-induced anorexia.

1- INTRODUÇÃO

Perspectiva Histórica da Teoria das Doenças Geradas por Citoquinas

Durante mais de 1500 anos, a doutrina médica atribuiu grande parcela do desenvolvimento das doenças à teoria humoral. De acordo com esta teoria proposta por Galeno (Claudius Galenus de Pergamum, 129-200 dC), o equilíbrio entre funções do coração, do cérebro, do fígado e do baço era responsável pelo bem estar das pessoas. Galeno acreditava que as doenças eram causadas pelo desequilíbrio dos humores corporais circulantes. Esta teoria não foi contestada até os avanços da microscopia e da microbiologia, que permitiu Rudolf Virchow formular a teoria da patologia celular e a Louis Pasteur desenvolver a teoria das doenças provocadas por organismos exógenos. Durante muito tempo, as pesquisas se concentraram em investigar as doenças causadas por fatores exógenos, sem tanta preocupação na investigação das causas endógenas. A observação de Pasteur no seu leito de morte quando disse “The germ is nothing, the terrain is everything”, deixou a falsa idéia de que os organismos exógenos eram os principais responsáveis pelo desenvolvimento das doenças. Por outro lado nesta mesma época, Claude Bernard, criou o conceito de homeostase e definiu que para o bom estado de saúde era necessário o equilíbrio do “milieu interieur” (ambiente interno) e mantido “por uma compensação contínua e delicada, estabelecida por reguladores muito sensíveis” (lições dos fenômenos da vida comuns aos animais e aos vegetais).

O senso comum na década de setenta ditou que a resposta imune aos patógenos ou a algum tipo de injúria era uniformemente uma resposta positiva do organismo. Acreditava-se que a resposta imune era responsável somente pela erradicação e/ou pela neutralização dos agentes invasores responsáveis pelo processo patológico. Assim, essa idéia sugeriu que o corpo jamais utilizaria suas próprias células para produzir moléculas tóxicas em quantidade suficiente para causar dano a algum tecido ou até mesmo a morte. O desenvolvimento do choque hemorrágico, do dano causado aos tecidos, do colapso circulatório, da necrose hemorrágica, da falência múltipla de órgãos, e de outros distúrbios associados com a infecção bacteriana, foram atribuídos à ação direta de toxinas patogênicas e não a resposta do hospedeiro. Mais tarde no início da década de oitenta, alguns estudos que exploraram mecanismos fisiopatológicos da febre, da perda do peso, das respostas de fase aguda à infecção aguda e crônica conduziram a uma mudança no entendimento da

patogênese de algumas doenças, demonstrando que proteínas produzidas por macrófagos e por outras células imunes, não patogênicas, seriam capazes de causar algumas síndromes e danos a diferentes tecidos em diferentes modelos experimentais. Essas proteínas, que posteriormente foram denominadas citocinas (que incluem por exemplo, IL-1 β , IL-6, e TNF- α), são conhecidas por produzirem respostas biológicas distintas que podem ser benéficas ou prejudiciais (Tracey, 2007).

Desta forma, emergiu a teoria das doenças provocadas por citocinas, determinando que as citocinas produzidas pelo sistema imune podem causar os sinais, os sintomas, e também respostas que podem ser patológicas (figura 1). Para citar um exemplo, durante choque séptico agudo induzido por bactérias Gram-negativas, altos níveis de TNF- α são produzidos (Tracey *et al.*, 1987). A administração de TNF- α em mamíferos saudáveis (incluindo seres humanos) reproduz as manifestações hemodinâmicas, metabólicas, imunológicas, e patológicas da doença (Tracey *et al.*, 1986), de maneira que o *knockout* do gene ou o bloqueio farmacológico da atividade do TNF- α impedem o desenvolvimento de choque séptico letal (Pfeffer *et al.*, 1993). Outras atividades fisiopatológicas atribuídas ao TNF- α são: a capacidade causar febre, inflamação localizada e anorexia (Dinarello *et al.*, 1986; Tracey *et al.*, 1986; Pfeffer *et al.*, 1993; Gayle *et al.*, 1998). Nos anos noventa, estudos clínicos demonstraram que o uso de inibidores específicos do TNF- α em pacientes com doenças inflamatórias promoveu uma melhora significativa na qualidade de vida dos pacientes, evidenciando o papel patogênico desta citocina (Elliott *et al.*, 1994; Targan *et al.*, 1997; Dinarello, 2004) e validando nos seres humanos a premissa fundamental da teoria das doenças geradas por citocina.



Figura 1

O Sistema Nervoso Central no Controle da Ingestão Alimentar:

Transmissão do Sinal de Insulina e Leptina

A leptina é expressa principalmente no tecido adiposo e em menores quantidades no epitélio gástrico e placenta (Maffei *et al.*, 1995; Masuzaki *et al.*, 1997; Bado *et al.*, 1998). A administração de leptina a camundongos *ob/ob* resulta em diminuição da ingestão alimentar, perda de peso e redução dos níveis glicêmicos (Campfield *et al.*, 1995), além de aumentar a atividade simpática em tecido adiposo marrom, com conseqüente aumento do gasto energético (Pelleymounter *et al.*, 1995).

Os níveis séricos de leptina correlacionam-se de forma positiva com o índice de massa corporal na grande maioria das populações estudadas (Frederich *et al.*, 1995; Maffei *et al.*, 1995; Considine *et al.*, 1996; Havel *et al.*, 1998). A secreção desse hormônio diminui com o jejum prolongado e estímulo β -adrenérgico (Ahima *et al.*, 1996) e aumenta em resposta à administração de insulina e glicocorticóides (De Vos *et al.*, 1995; Saladin *et al.*, 1995). A leptina é secretada de forma pulsátil e inversamente relacionada à

atividade do eixo ACTH-Cortisol, ou seja, ocorre diminuição da secreção de leptina ao amanhecer e aumento no final da tarde (Licinio & Wong, 1997).

A leptina produzida pelo tecido adiposo informa o estado nutricional do indivíduo a centros hipotalâmicos, que regulam a ingestão alimentar e o gasto energético. Assim, a redução da quantidade de tecido adiposo leva a diminuição dos níveis circulantes de leptina, estimulando a ingestão alimentar e reduzindo o gasto energético. Contrariamente, o aumento do estoque de tecido adiposo está associado à elevação dos níveis séricos de leptina, diminuindo a ingestão alimentar e aumentando o gasto energético (Schwartz *et al.*, 2000).

O receptor de leptina (OBR) é membro da família gp130 da classe I dos receptores de citocinas (Tartaglia *et al.*, 1995). É encontrado em muitos tecidos com várias formas de *splicing*, sendo as mais encontradas a forma curta (OBR_S), expressa em vários tecidos, que apresenta domínios intracelulares truncados, e a forma longa (OBR_L), que apresenta domínios intracelulares longos e é expressa principalmente no hipotálamo (núcleos paraventricular, arqueado, ventromedial e dorsomedial) (Mercer *et al.*, 1996; Woods *et al.*, 1996). O OBR_S não tem sua função bem definida, mas parece influir no transporte da leptina através da barreira hematoencefálica e talvez contribua para a depuração da leptina atuando como uma fonte de receptor solúvel.

Inter-relações e Implicações Fisiopatológicas

A homologia do receptor de leptina à classe I dos receptores de citocinas forneceu informações importantes para a descoberta dos possíveis mediadores intracelulares da ação da leptina. Os receptores da classe I das citocinas agem através das famílias das proteínas JAK (Janus Quinase) e STAT (*Signal Transducers Activators of Transcription*) (Heldin, 1995). Tipicamente, as proteínas JAK estão constitutivamente associadas com seqüências de aminoácidos dos receptores, e adquirem sua atividade tirosina quinase após a ligação do hormônio a seu receptor. Uma vez ativada, a proteína JAK fosforila o receptor induzindo a formação de um sítio de ligação para as proteínas STAT, as quais são ativadas após terem se associado ao receptor e serem fosforiladas pela JAK. As proteínas STAT ativadas são translocadas para o núcleo e estimulam a transcrição

(Zhang & Scarpance, 2006). No entanto a homologia do receptor de leptina à classe I dos receptores de citocinas, permite que várias outras citocinas amplifiquem a transmissão do sinal da leptina. Assim as proteínas subseqüentes ao receptor de leptina (JAK e STAT) podem exercer uma interface no controle da ingestão alimentar, regulando fatores de saciedade e adiposidade à longo prazo (através da própria leptina) ou desenvolvendo sinais anorexigênicos patológicos (através de citocinas) (Plata-Salaman, 1996).

O receptor de leptina também é capaz de estimular outras vias de sinalização além da JAK/STAT, tais como a via da proteína quinase ativadora de mitose (MAPK) e a via de fosfatidilinositol 3-quinase (PI 3-quinase) (Heldin, 1995), e é possível que a capacidade do OBR controlar o peso dependa também destas vias de sinalização (Tartaglia, 1997; Niswender *et al.*, 2001).

Assim como a leptina, a insulina também é considerada um hormônio que sinaliza ao hipotálamo o estoque de tecido adiposo e modula a ingestão alimentar (Woods *et al.*, 1979; Woods *et al.*, 1985). A insulina circula em níveis proporcionais ao conteúdo de tecido adiposo e atravessa a barreira hematoencefálica via um sistema de transporte saturável em níveis proporcionais aos plasmáticos (Baura *et al.*, 1993). Os receptores de insulina são expressos por neurônios envolvidos na ingestão alimentar (Baskin *et al.*, 1988; Cheung *et al.*, 1997; Baskin *et al.*, 1999). A administração de insulina no sistema nervoso central reduz a ingestão alimentar e diminui o peso corporal, enquanto a deficiência desse hormônio causa hiperfagia (Sipols *et al.*, 1995).

A correlação dos níveis séricos de insulina com o conteúdo de gordura corporal é conseqüência da resistência à insulina induzida pelo aumento da gordura corporal (Schwartz *et al.*, 1997). Assim, à medida que o peso corporal aumenta a insulina deve aumentar para compensar a resistência à insulina e manter a homeostase de glicose (Polonsky *et al.*, 1988; Kahn *et al.*, 1993).

A Modulação de Neuropeptídeos Hipotalâmicos e o Controle da Fome

O hipotálamo foi implicado pela primeira vez como responsável pela estabilidade do ganho de massa corporal a cinquenta anos atrás. Investigações lesionando e estimulando alguns núcleos hipotalâmicos definiram inicialmente que o núcleo

ventromedial (VMH) representava o “centro da saciedade” e o núcleo hipotalâmico lateral (LHA) como um “centro da fome” (Stellar, 1994). Além da descoberta de que núcleos hipotalâmicos específicos eram responsáveis pelo controle da homeostase energética, mais tarde, um novo conceito foi criado identificando que esta regulação acontece por circuitos neuronais, os quais utilizam neuropeptídeos específicos. O núcleo arqueado (ARC), em particular, é cogitado, como sendo o local onde acontece a interação dos sinais envolvidos na regulação do apetite (Elmquist *et al.*, 1999). Esse núcleo é acessível aos sinais de balanço energético provenientes da circulação, transportadas através da barreira hematoencefálica e órgãos circunventriculares do cérebro (Broadwell & Brightman, 1976). O ARC está interconectado a outros núcleos hipotalâmicos também importantes no controle da ingestão alimentar (Elias *et al.*, 1998).

Após a ativação dos receptores de leptina e/ou insulina no cérebro e das proteínas envolvidas na transmissão dos sinais desses hormônios, respostas neuronais integradas são necessárias para modular a ingestão alimentar e o gasto energético (figura 2). A ingestão alimentar e o gasto energético são regulados em diferentes regiões do hipotálamo envolvendo uma complexa rede neuronal (Baskin *et al.*, 1999). O entendimento atual do sistema nervoso envolvido nesta regulação sugere que, no hipotálamo, há dois grandes grupos de neuropeptídeos envolvidos nos processos orexígenos e anorexígenos (Schwartz *et al.*, 2000). Dentre os neuropeptídeos orexigênicos envolvidos no funcionamento dessa rede neuronal que estimulam a ingestão alimentar destacam-se o neuropeptídeo Y (NPY) (Stephens *et al.*, 1995) e o Agouti related peptide (AgRP) (Shutter *et al.*, 1997), enquanto dentre os neuropeptídeos anorexigênicos responsáveis pela redução da ingestão alimentar estão o cocaine - and amphetamine- regulated transcription (CART) (Kristensen *et al.*, 1998) e o melanocyte stimulating hormone (a-MSH) (Fan *et al.*, 1997). Tanto a leptina como a insulina, regulam o balanço energético diminuindo os níveis de neuropeptídeos anabólicos NPY e AGRP e aumentando a concentração de neuropeptídeos catabólicos POMC, CART e α -MSH.

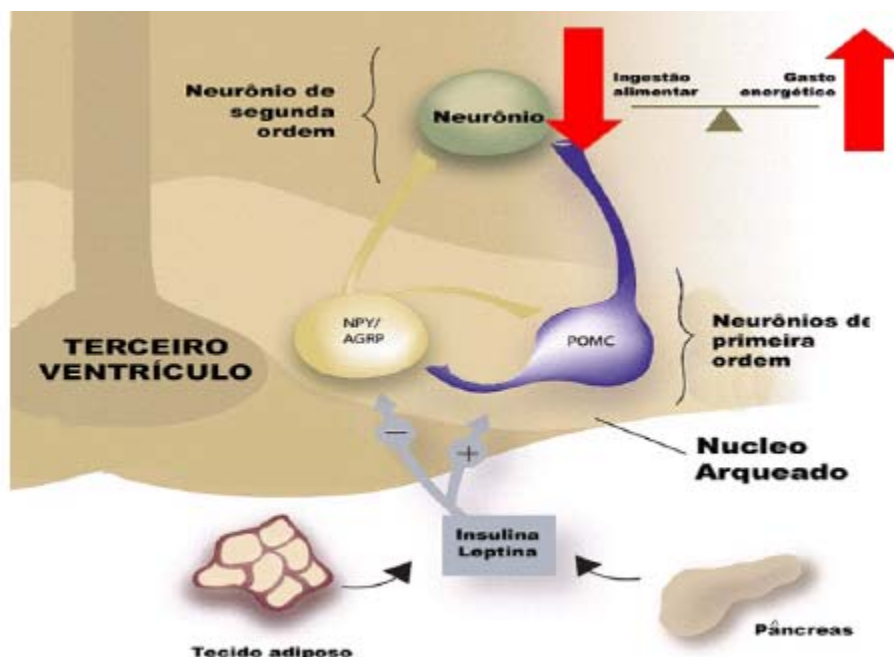


Figura 2

Os neurônios que expressam esses neuropeptídeos interagem com cada outro e com sinais advindos da periferia (como a insulina, leptina, grelina, etc), atuando na regulação do controle e do gasto energético (Flier, 2004). A localização precisa dos receptores neurais para cada sinal orexigênico e anorexigênico não está totalmente compreendida. Sabe-se, no entanto, que os receptores para estes sinais estão presentes principalmente no núcleo arqueado (ARC) e paraventricular (PVN) hipotalâmico, mas eles não estão restritos a estas áreas (Wynne *et al.*, 2005).

O Desenvolvimento da Síndrome Anorexia-Caquexia

A caquexia é um estado involuntário de perda de peso encontrado em doenças neoplásicas, infecciosas e inflamatórias que contribui marcadamente para a mortalidade dessas moléstias. A anorexia é um dos principais indutores de caquexia, embora o padrão

de perda de peso observado na caquexia difira daquele observado na restrição alimentar (Tisdale, 1997).

A palavra caquexia deriva do grego “kakos” que significa “mal” e “hexis” que quer dizer “condição” (Tartaglia, 1997). Aproximadamente metade dos pacientes com câncer desenvolve caquexia, caracterizada por anorexia e diminuição do tecido adiposo e massa muscular. Em geral, pacientes com tumores sólidos têm uma maior frequência de caquexia (Bruera, 1997). No momento do diagnóstico, aproximadamente 80% dos pacientes com câncer do trato gastrointestinal e 60% dos pacientes com câncer de pulmão têm perda de peso significativa. A caquexia é mais comum em crianças e idosos e se torna mais pronunciada com o evoluir da neoplasia.

A síndrome anorexia-caquexia é desencadeada por uma inter-relação complexa de variáveis metabólicas e comportamentais que se correlacionam com prognósticos ruins e comprometimento da qualidade de vida dos pacientes (Bruera, 1997; Tisdale, 1997; Larkin, 1998). Apesar da etiologia da caquexia não ser bem definida, várias hipóteses têm sido exploradas incluindo a participação do óxido nítrico, de citocinas, hormônios circulantes, neuropeptídeos, neurotransmissores e fatores derivados dos tumores (Schwartz *et al.*, 1995; Plata-Salaman, 1997; Larkin, 1998). Entretanto, a hipótese de que a síndrome anorexia-caquexia seja causada pela ação de moléculas pró-inflamatórias produzidas pelo câncer ou liberadas pelo sistema imune em resposta à presença do tumor no sistema nervoso central (SNC) tem ganhado destaque nos últimos anos.

Dentre os agentes que atuam no SNC responsáveis pelo desenvolvimento da anorexia durante o desenvolvimento tumoral, o óxido nítrico (NO) parece ter participação determinante (Laviano *et al.*, 2003). O NO é uma molécula gasosa multifuncional e um radical livre altamente reativo. Ele é sintetizado a partir da L-arginina, NADPH e oxigênio por enzimas denominadas óxido nítrico sintetase (NOS) (Gaston *et al.*, 2003). Como molécula sinalizadora, o NO regula diversos processos fisiológicos e fisiopatológicos, como funções vasculares, neurológicas e, em concentrações relativamente elevadas, citotóxicas. Muitas das funções da molécula baseiam-se na habilidade do NO em alterar a função de proteínas através de modificações pós-traducionais. Em mamíferos, o NO é sintetizado pela família de enzimas conhecida como óxido nítrico sintases (NOS)

(Knowles & Moncada, 1994). A síntese do NO é catalisada por uma das três isoformas de NOS: NOS1 (neuronal ou nNOS), NOS2 (induzida ou iNOS) e NOS3 (endotelial ou eNOS) (Knowles, 1996).

O gene da iNOS está localizado no cromossomo 17 (Xu *et al.*, 1994). iNOS murina e humana apresentam aproximadamente 80% de similaridade em suas seqüências (Taylor & Geller, 2000). Diferentemente das outras duas isoformas constitutivas, iNOS não é dependente de Ca^{2+} para exercer suas funções. Calmodulina permanece ligada de forma não-covalente ao complexo iNOS e constitui, assim, uma sub-unidade essencial dessa isoforma. Atividade da iNOS foi demonstrada em diversas células e tecidos (Stuehr & Griffith, 1992). A expressão do gene da iNOS e subsequente translação do RNAm é estimulada por um grande número de substâncias, principalmente endotoxinas e mediadores pró-inflamatórios. O aumento das concentrações da iNOS provenientes do tecido tumoral, é capaz de alcançar o SNC e contribuir para o aumento de citocinas pró-inflamatórias em regiões do hipotálamo responsáveis pelo controle da homeostase energética (Laviano *et al.*, 2003). As principais citocinas envolvidas neste processo são fator de necrose tumoral α (TNF- α), interleucina 1 β (IL-1 β) e interferon γ (IFN- γ), assim é cada vez maior o interesse no papel da iNOS nas vias de transdução de sinais que controlam a ingestão alimentar durante a anorexia induzida por tumor.

Postula-se que numerosas citocinas (TNF- α , IL-1 β , IL-6, IFN- γ) participem da síndrome anorexia-caquexia observada em pacientes com câncer. As citocinas podem ser liberadas na circulação e transportadas para o cérebro através da barreira hematoencefálica e órgãos circunventriculares (áreas que permitem a passagem mais fácil de moléculas na barreira hematoencefálica), como é o caso da IL-6. Por outro lado, as citocinas também são produzidas por neurônios e células da glia do SNC, em resposta ao aumento das citocinas periféricas (Hopkins & Rothwell, 1995; Licinio & Wong, 1997; Sternberg, 1997; Haslett, 1998; Mantovani *et al.*, 1998). Embora o local da síntese de citocinas no SNC seja dependente da natureza do estímulo, as doenças sistêmicas parecem influenciar predominantemente a atividade hipotalâmica, área com a maior densidade de receptores para a maioria das citocinas (Hopkins & Rothwell, 1995).

Altos níveis circulantes de IL-1 β , TNF- α e IL-6 são encontrados em alguns, mas não todos, pacientes com câncer, e os níveis dessas citocinas se correlacionam com a progressão do tumor (Moldawer *et al.*, 1992; Noguchi *et al.*, 1996; Matthys & Billiau, 1997). A administração crônica dessas citocinas, tanto isoladamente como em conjunto são capazes de reduzir a ingestão alimentar e reproduzir as diferentes características da síndrome anorexia-caquexia (Gelin *et al.*, 1991; Moldawer *et al.*, 1992; Matthys & Billiau, 1997; Tisdale, 1997; Mantovani *et al.*, 1998). Outras evidências do envolvimento dessas citocinas na regulação do peso corporal no hipotálamo foram adquiridas através de estudos com neutralização específica das diversas citocinas através de anticorpos (Sherry *et al.*, 1989; Noguchi *et al.*, 1996; Matthys & Billiau, 1997).

Dentre as citocinas pró-inflamatórias envolvidas no desenvolvimento da anorexia, a IL-1 β tem ganhado destaque nos últimos anos. Esta molécula é rapidamente liberada em resposta a infecção ou inflamação e causa anorexia, febre, sonolência e letargia (Kent *et al.*, 1996; Dantzer, 2001). A administração intracerebroventricular do antagonista de IL-1 β atenua os efeitos da redução do apetite causados pela injeção de IL-1 β (Kent *et al.*, 1992), sugerindo que a IL-1 β estimula sua própria produção no SNC ou que a IL-1 β possa cruzar a barreira hematoencefálica para alcançar seus efeitos. A IL-1 β também pode influenciar o funcionamento do SNC através do aumento da atividade do nervo vago (Watkins *et al.*, 1995; Bluthé *et al.*, 1996) e da indução local da molécula de sinalização (ex. óxido nítrico e prostaglandinas) por células da microvasculatura cerebral na barreira hematoencefálica (Van Dam *et al.*, 1996) e nas estruturas circunventriculares do cérebro (Blatteis, 1992). Alguns estudos apontam a ação anorética da IL-1 β no hipotálamo aconteça no núcleo arqueado. Este núcleo hipotalâmico é um dos poucos núcleos celulares do SNC de ratos onde os receptores de IL-1 β parecem estar expressos (Ericsson *et al.*, 1995) e são ativados em resposta a administração de IL-1 β (Herkenham *et al.*, 1998). Em adição aos vários meios indiretos pelos quais a IL-1 β pode influenciar o núcleo arqueado, existem evidências de que esta citocina possa alcançá-lo diretamente. A parte medial do núcleo arqueado, onde o receptor de IL-1 β (IL-1R1) é expresso, pode se ligar a alguns peptídeos e proteínas administrada por via intravenosa (van Houten *et al.*, 1981) uma vez que tem como eminência mediana (estrutura circunventricular) onde a permeabilidade capilar é bem

maior que das regiões adjacentes (Shaver *et al.*, 1992). Entretanto, os mecanismos moleculares no hipotálamo responsáveis pela redução da ingestão alimentar induzida pela ação central das citocinas, ainda são amplamente desconhecidos.

Apetite, AMP-kinase, e Malonil-CoA

Recentemente, três grupos descreveram de forma independente que a proteína quinase ativada por AMP (AMPK), a chave mestra do controle da homeostase energética intracelular (Hardie & Carling, 1997), poderia também regular a ingestão alimentar (Andersson *et al.*, 2004; Kim *et al.*, 2004b; Minokoshi *et al.*, 2004), i.e., a enzima que determina o destino dos nutrientes ingeridos poderia também regular a quantidade da ingestão alimentar. De acordo com esses os primeiros estudos, a leptina inativa (desfosforila) a AMPK no hipotálamo, enquanto Kim *et al.* (Kim *et al.*, 2004a) descreveram que o C75, um composto anorexigênico, faz o mesmo. Uma vez que a leptina ativa a AMPK no músculo esquelético (Minokoshi *et al.*, 2002) e provavelmente em outros tecidos extraneurais, a inativação da AMPK no hipotálamo parece um contra-senso, talvez isso seja reflexo de uma complexa rede de integração entre neurônios sensíveis à leptina (Pinto *et al.*, 2004). Outros mediadores de anorexia como a injeção intracerebroventricular de insulina ou glicose e a realimentação também resultaram na inativação da AMPK e conseqüente redução da ingestão alimentar (Andersson *et al.*, 2004). Por outro lado, fatores orexigênicos como o AICAR e a grelina fosforilam (ativam) a AMPK hipotalâmico que fosforila (inativa) o Acetil CoA carboxilase (ACC), reduzindo a produção de malonil-CoA e aumentando a ingestão alimentar (Andersson *et al.*, 2004).

A inativação da AMPK reduz a fosforilação da ACC a qual aumenta sua atividade e aumenta a produção de malonil CoA (figura 3). Previamente (Loftus *et al.*, 2000) propuseram que a malonil CoA reduz o apetite.

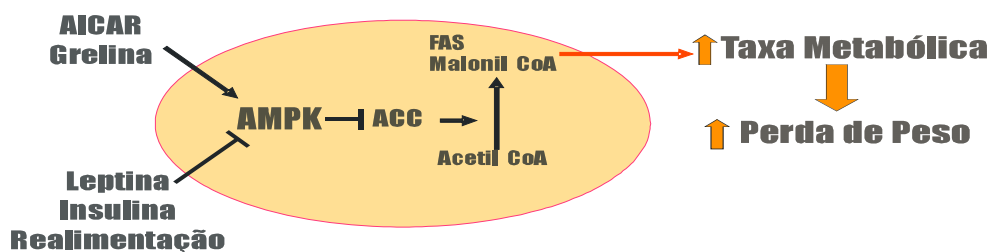


Figura 3

AMPK e a Inflamação

AMPK é uma molécula heterotetramérica composta de uma subunidade catalítica α e duas subunidades regulatórias β e γ . Esta proteína é ativada pela fosforilação do resíduo de treonina 172 da alça de ativação da subunidade α (Hardie & Carling, 1997). A ativação é causada pelo aumento da relação AMP:ATP secundário à redução da quantidade de ATP intracelular ou através de quinases tais como o complexo AMPK-quinase.

Identificada em diferentes tecidos como o músculo esquelético, fígado, coração e hipotálamo, a AMPK exerce funções distintas como captação de glicose, oxidação de AG, produção hepática de glicose dentre outras (Kahn *et al.*, 2005), no entanto é crescente o número de evidências experimentais que apontam propriedades anti-inflamatórias da AMPK. A ativação farmacológica desta enzima com o 5-amino-4-imidazole carboxamida ribose (AICAR), foi capaz de bloquear o processo inflamatório mediado por lipopolissacarídeo (LPS) em cultura primária de astrócitos, microglia, células da glia e macrófagos de ratos (Giri *et al.*, 2004; Jhun *et al.*, 2004). Além disso, a ativação hipotalâmica da AMPK inibiu a expressão de iNOS, IL-1 β e TNF- α no córtex cerebral de roedores que receberam injeção intraperitoneal de LPS (Giri *et al.*, 2004). O mecanismo responsável pela redução da expressão de citocinas pró-inflamatórias mediado pela AMPK ainda não está completamente elucidado. Hattori e colaboradores, sugeriram que a ativação da AMPK pela metformina reduz a inflamação através da menor degradação

citoplasmática do I κ B e consequentemente pela redução da atividade transcripcional do Fator Nuclear κ B (NF- κ B) (Hattori *et al.*, 2006). Além disso, outro estudo apontou que a fosforilação da AMPK em células da glia atenuou a atividade da CCAAT/*enhancer-binding protein* (C/EBP) (Giri *et al.*, 2004) que ao se ligar ao DNA em regiões promotoras específicas, ativa a transcrição gênica de moléculas pró inflamatórias importantes. Embora muitas evidências apontem a importância da AMPK no controle do processo anti-inflamatório (*in vitro* e *in vivo*), da ingestão alimentar e no controle do peso corporal em diferentes modelos de animais, o papel da AMPK neuronal durante o desenvolvimento da anorexia induzida pelo câncer ainda é totalmente desconhecida.

O Tumor de Walker

A linhagem celular do tumor de Walker é derivada de câncer de mama e foi descoberta isolada em 1928 por George Walker. O tumor de Walker-256 tem sido amplamente utilizado como um modelo experimental para indução de caquexia em ratos principalmente devido ao fácil transplante e por ser espécie específica para ratos (Fernandes *et al.*, 1994).

Esse tumor cresce rapidamente e promove alterações significativas nos níveis plasmáticos de hormônios e citocinas (Siddiqui & Williams, 1990; Waki *et al.*, 1999). A presença do tumor aumenta os níveis plasmáticos de glicocorticóides e reduz a insulinemia. A produção de interleucinas é aumentada na presença do tumor e postula-se que essas citocinas sejam as principais responsáveis pela caquexia induzida por esse tumor (Siddiqui & Williams, 1989).

Apesar da importância das citocinas no desenvolvimento da síndrome anorexia-caquexia pouco se conhece a respeito das vias intracelulares que modulam o apetite no hipotálamo. Assim o objetivo desse projeto foi caracterizar a participação no desenvolvimento de caquexia da AMPK no hipotálamo de ratos com tumor de Walker-256.

2- OBJETIVOS

- Caracterizar a fosforilação da AMPK no hipotálamo de ratos com tumor de Walker-256.
- Investigar os efeitos da administração de AICAR (i.c.v.) e 2DG (i.p.) sobre a ativação da AMPK hipotalâmica, ingestão alimentar e sobrevida de ratos com anorexia induzida por tumor.
- Determinar se a ativação da AMPK reduz a resposta inflamatória no hipotálamo de ratos com tumor de Walker-256.
- Determinar se a ativação da AMPK modula a expressão de neuropeptídeos em ratos com tumor de Walker-256.
- Determinar se a administração oral de metformina ativa AMPK em hipotálamo de ratos com tumor de Walker-256.
- Investigar a influência da administração da metformina sobre a ingestão alimentar e na sobrevida de ratos com anorexia induzida por tumor.

3- CAPÍTULO

A CENTRAL ROLE FOR HYPOTHALAMIC AMP ACTIVATED PROTEIN KINASE (AMPK) IN CANCER-INDUCED ANOREXIA

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Short running title: Activation of hypothalamic AMPK reversed anorexia in rats.

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ABSTRACT

The pathogenesis of cancer anorexia is multifactorial and is associated with disturbances of the central physiological mechanisms controlling food intake. However the neurochemical mechanisms responsible for cancer induced-anorexia are unclear. Here, we show that chronic infusion of 5-amino-4imidazolecarboxamide-ribose (AICAR) into the third cerebral ventricle and a chronic peripheral injection of 2 deoxy-D-glucose (2DG) promotes hypothalamic AMPK activation, increased food intake and prolongs the survival of anorexic tumor bearing (TB) rats. In parallel, the pharmacological activation of hypothalamic AMPK in TB animals, markedly reduced the hypothalamic production of inducible nitric oxide synthase (iNOS), interleukin-1 β IL-1 β and tumor necrosis factor-alpha (TNF- α) and modulated the expression of hypothalamic neuropeptides (NPY and POMC) involved in the control of energy homeostasis. Furthermore, the daily oral treatment with biguanide anti diabetic drug metformin also induced AMPK phosphorylation in the central nervous system and increased food intake and life span in anorexic TB rats. Collectively, the findings of this study suggest that hypothalamic AMPK activation reverses cancer anorexia by inhibiting the production of proinflammatory molecules and controlling the neuropeptide expression in the hypothalamus, reflecting in a prolonged life span in TB rats. Thus, our data indicate that hypothalamic AMPK activation presents an attractive opportunity for the treatment of cancer-induced anorexia.

INTRODUCTION

Approximately 50% of cancer patients report abnormalities in eating behavior upon diagnosis (1). Anorexia and reduced energy intake also negatively affect quality of life, which is an important endpoint in the management of patients with cancer and in the design of clinical trials (2). Accumulating evidence indicates that cancer anorexia is multifactorial in its pathogenesis, and most of the hypothalamic neuronal signaling pathways modulating energy intake are likely to be involved (3).

Tumour growth is frequently associated with the development of anorexia (3). Evidence suggests that multiple proinflammatory-cytokines [Interleukin-1 β (IL-1 β , Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α)] in the central nervous system are mediators of anorexia in tumor-bearing hosts (4). Several studies have sought to explain how peripheral cytokines enter the brain and affect neuronal pathways involved in food intake regulation (5, 6). These cytokines may play a pivotal role in long-term inhibition of feeding by mimicking the hypothalamic effect of excessive negative feedback signaling from leptin (3). This could be done by persistent stimulation of anorexigenic neuropeptides, such as corticotropin-releasing factor, as well as by inhibition of the neuropeptide Y (NPY) orexigenic network (7).

Analogous to leptin, pro-opiomelanocortin (POMC)-containing neurons in the ARH also contain receptors to cytokines such as IL-1 β and leukemia inhibitory factor, and the injections of each of these molecules into the third ventricle up-regulates POMC, and decreases feeding behavior (8). In addition, the high levels of IL-1 β , IL-6, and TNF- α interfere with NPY release and neurotransport to a greater degree than gene expression. Some studies have reported that hypothalamic mRNA and peptide levels of orexigen NPY are decreased in different models of anorectic tumor-bearing rats (9, 10).

Multiple hypothalamic neuronal signaling pathways and the cross-talk between these pathways are involved in the control of energy intake (11-13). Recently, hypothalamic AMP-activated protein kinase (AMPK) signaling has become an important focus of interest in the control of food intake. It has been proposed that changes in neuronal energy status affect AMPK phosphorylation and neuropeptide expression, leading to

changes in food intake (14-16). AMPK is the central component of a protein kinase cascade that plays a key role in the regulation of energy control. AMPK is activated in response to ATP depletion, which causes an increase in the AMP:ATP ratio (17, 18). AMPK activation occurs through the phosphorylation of specific threonine residue (Thr-172), catalyzed by an upstream kinase that has been identified as LKB1 (19).

Growing experimental evidences suggests the innumerable anti-inflammatory effects of AMPK; since, the pharmacological activation of AMPK with 5-amino-4-imidazole carboxamide ribose (AICAR), blocks LPS-induced inflammatory processes by blocking the expression of proinflammatory cytokines, iNOS, COX-2 and MnSOD genes in astroglial cells (20, 21). Furthermore, the activation of AMPK inhibits LPS-induced expression of proinflammatory cytokines in primary rat astrocytes, microglia and peritoneal macrophages (20, 22). Although growing evidence demonstrates the importance of AMPK activity in regulating the inflammatory process (*in vitro* and *in vivo*) and food intake and body weight in different models of animals, the role of neuronal AMPK activity in cancer-induced anorexia is not known.

The present data demonstrate that different pharmacological activators of AMPK signaling, including oral administration of the anti diabetic drug metformin, increases AMPK phosphorylation in the central nervous system, reverses cancer anorexia and increases the life span in TB rats through the inhibition of the production of proinflammatory molecules and by controlling neuropeptide expression in the hypothalamus.

MATERIALS AND METHODS

Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad. Tris[hydroxymethyl]amino-methane (Tris), aprotinin, ATP, dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, Tween 20, glycerol, and bovine serum albumin (fraction V) were from Sigma Chemical Co. (St.Louis, MO). Nitrocellulose paper (BA85, 0.2mm) was from Schleicher & Schuell (Keene, NH).

Ketamine hydrochloride was from Cristália (Itapira SP, Brazil). Antiphospho-[Ser⁷⁹] ACC was from Upstate Biotechnology (Charlottesville, VA, USA). Anti-ACC, and anti-iNOS were from Santa Cruz Biotechnology, Inc. Antiphospho-[Thr¹⁷²] AMPK and anti-AMPK were from Cell Signaling Technology (Beverly, MA, USA). 5-amino-4imidazolecarboxamide-ribose (AICAR), 2-deoxy-D-glucose (2DG) and metformin (1,1-dimethylbiguanide hydrochloride) were obtained Sigma- Aldrich, (St. Louis, MO, USA) and were dissolved in sterile saline. The doses administrated in each experimental group are given below.

Animals and Surgical Procedures

Adult male Wistar rats (250–300 g) were used in all of the experiments in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA); the ethics committee at the State University of Campinas approved experiments. Room temperature was maintained (28±1 °C), and rats were housed in individual cages, subjected to a standard light-dark cycle (6:00 a.m. to 6:00 p.m./6:00 p.m. to 6:00 a.m.), and provided with standard rodent chow and water ad libitum. After an overnight fast, the rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and positioned on a Stoelting stereotaxic apparatus. The implantation of an intracerebroventricular (i.c.v.) catheter into the third ventricle has been previously described (23). After a 1-wk recovery period, cannula placement was confirmed by a positive drinking response after administration of angiotensin II (40 ng/2 µl), and animals that did not drink 5 ml of water within 15 minutes after treatment were not included in the experiments.

The Walker 256 tumor cell line (originally obtained from the Christ Hospital Line, National Cancer Institute Bank, Cambridge, USA) is currently maintained frozen in liquid nitrogen in our laboratory. For experimental procedures, Walker 256 cells were maintained through consecutive intraperitoneal inoculation, each with 2×10^6 tumor cells in PBS (1 ml). Walker 256 tumor cells were isolated from ascitic fluid 4 days after inoculation, and used when the cell viability was > 98%, as estimated by the trypan blue exclusion method. The rats received a subcutaneous injection of Walker 256 tumor cells (approximately 2×10^6 cells in 1 ml saline solution) in the right flank after cannulation surgery.

Definition of cancer anorexia and treatments

The metabolic and feeding experiments were performed after complete recovery from the operation according to the protocol depicted in figure 1. Each animal's individual baseline 24 h food intake was defined as the average daily food intake over a period of 3 days, following recovery from cannulation surgery. Subsequent food intake data are expressed as a percent of individual, baseline daily food intake. In TB animals, cancer anorexia was defined as a single value below 70% of baseline occurring after a steady decline of at least 3 d duration. Data are presented as group averages of the percent of individual baseline food intake.

When criteria for anorexia had been met, TB animals were daily treated with: i.p. injection of vehicle or 2DG (500mg/kg); i.c.v. injection (3 μ l bolus injection) of vehicle or AICAR (0.5 or 2.0 mM); or oral gavage with metformin (500 mg/kg). These treatments were performed during 4 days prior to Western Blott and cumulative food intake analysis and the daily chronic treatment was conducted for survival curve determination.

Physiological and metabolic parameters

After six hours of fasting, rats were submitted to an insulin tolerance test (ITT; 1U/kg body weight of insulin). After 30 minutes of i.c.v. AICAR, 180 minutes of i.p. 2DG and 180 minutes of oral metformin administration, the rats were injected with insulin and the blood samples were collected at 0, 4, 8, 12 and 16 minutes from the tail for serum glucose determination. The rate constant for plasma glucose disappearance (Kitt) was calculated using the formula $0.693 / (t_{1/2})$. The plasma glucose $t_{1/2}$ was calculated from the slope of last square analysis of the plasma glucose concentration during the linear phase of decline (24). Plasma glucose was determined using a glucose meter (Roche Diagnostic, Rotkreuze, Switzerland) and RIA was employed to measure serum insulin, according to a previous description (25). Leptin concentrations were determined using a commercially available Enzyme Linked Immuno Sorbent Assay (ELISA) kit (Crystal Chem Inc, Chicago, IL).

Proinflammatory-cytokine determination.

IL1 β and TNF- α were measured in the hypothalami of controls, treated and untreated TB animals rats using ELISA kits for IL1 β and TNF- α (Pierce Endogen, Rockford, IL, USA), according to manufacturer's instructions.

mRNA isolation and RT-PCR

NPY and POMC neuropeptides were measured in the hypothalami of controls, treated and untreated TB animals rats under fasting conditions (12 hours). Seven micrograms of total RNA were reverse-transcribed with superscript reverse transcriptase (200 U/ μ l) using oligo (dT) (50mmol/L) in a 30 μ l reaction volume (5 x RT buffer, 10 mmol/L dNTP and 40 U/ μ l RNase free inhibitor). The reverse transcription involved a 50-min incubation at 42°C and a 15 incubation at 70°C. The RTP 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 (Nucleo Tech, San Mateo, CA, USA) and band quantification was performed using the Gel Expert Software (Nucleo Tech). 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 neuropeptide Y (NPY) and proopiomelanocortin (POMC) were 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'-AGGCAAGATGGGTCACCAGC-3', antisense: 5'-AGTCGAATCATCCATTCAGGTCG-3' (fragment: 202 bp; T_m : 57°C; amplification: 27 cycles); NPY (NCBI: NM012614), sense: 5'-AGAGATC CAGCCCT GAGACA-3', antisense: 5'- AACGACAACAAGGGAAATGG-3' (fragment: 236 bp; T_m : 62; amplification: 31 cycles); POMC (NCBI: AF510391), sense: 5'-CTCCTGCTTCAGACCTCCAT-3', antisense: 5'-TTGGGGTACACCTTCACAGG-3' (fragment: 398 bp; T_m : 63; amplification: 32 cycles).

Immunoblotting

Four days after the respective treatments, rats were anaesthetized with sodium amobarbital (15 mg/kg body weight, i.p.) and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The rats were killed and hypothalami were removed at the time-points indicated, minced coarsely, and homogenized immediately in solubilization buffer containing (mM) 100 Tris (pH 7.6), 1% Triton X-100, 10 Na₃VO₄, 100 NaF, 10 Na₄P₂O₇, 4 EDTA, 150 NaCl, 0.1 mg aprotinin and 35 mg phenylmethylsulfonyl fluoride/mL, using a polytron PTA 20S generator (Model PT 10/35, Brinkmann Instruments, Westbury, NY) operated at maximum speed for 30 seconds and clarified by centrifugation. Invariably, hypothalami were pooled and 200 µg of protein were used for immunoblotting followed by Western blot analysis with the indicated antibodies. Blots were exposed to pre-flashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80 °C for 12–48 h. Band intensities were quantitated by optical densitometry (Scion Image software, ScionCorp).

Statistical Analysis

The survival curves were estimated using Kaplan-Meier's estimates, and curves were compared using the log-rank test. Where appropriate, results are expressed as the means $\pm$ S.E.M. accompanied by the indicated number of rats used in experiments. Comparisons among groups were made using parametric one-way ANOVA; where *F* ratios were significant, further comparisons were made using the *Bonferroni* test. The level of significance was set at $p < 0.05$. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry.

RESULTS

Physiological and metabolic parameters

Table 1 shows comparative data for controls and tumor bearing (TB) groups with respective treatments. As previously shown (26, 27), four days after the criteria of anorexia had been met, all the TB groups showed weight loss, reduction in the fasting serum insulin and leptin levels and insulin resistance when compared to age-matched

controls. When the tumor weights were excluded, treatments with intracerebroventricular (i.c.v.) AICAR, intraperitoneal (i.p.) 2DG and oral metformin were observed to increase the body weight, when compared to saline treatment in TB animals. In addition, AICAR, 2DG and metformin increased the fasting serum insulin and leptin and improved insulin sensitivity, compared with saline treatment in TB rats. No significant variations were found in fasting serum glucose levels between the groups and the tumor weights were similar between TB groups.

Central infusion of AICAR and peripheral injection of 2DG increase hypothalamic AMPK phosphorylation in TB rats.

We first examined the hypothalamic AMPK phosphorylation in tumor bearing (TB) and control rats under feeding and fasting states. Under feeding conditions, AMPK phosphorylation was similar in the hypothalamus of TB and control rats; however, in the fasting state the hypothalamic AMPK phosphorylation in the TB animals was reduced by 2.1- fold when compared to the control group (Fig. 2A-upper panel). The protein expression of AMPK was similar in the hypothalamus of controls and TB rats in both conditions (Fig. 2A-lower panel).

The i.c.v. microinfusion of AICAR and i.p. injection of 2DG in TB rats caused an increase in hypothalamic AMPK phosphorylation in a dose- and time-manner dependent (Fig. 2B and C). The i.c.v. infusion of AICAR (0.5 and 3.0 μ g) increased AMPK phosphorylation in the hypothalamus of TB rats by 2.2- and 4.9-fold at 60 minutes, respectively, when compared to vehicle (Fig. 2B-upper panel). The 2DG (250 and 500 mg/kg) i.p. injection increased the hypothalamic AMPK phosphorylation by 1.2- and 3.6-fold, respectively, in the hypothalamus of TB rats at 180 minutes, when compared to vehicle (Fig. 2C). The AMPK protein levels did not differ between the groups after AICAR or 2DG administration (Fig. 2B and C-lower panels).

AMPK activation attenuates cancer anorexia and increases survival in tumor bearing rats

To determine whether the hypothalamic AMPK activation increases food intake and survival in TB animals, we administered i.c.v. AICAR or i.p. 2DG during the period of anorexia in TB rats. Figures 3A and B show that the tumor development markedly reduced

food intake during 24 hours in TB rats after saline treatment when compared to control animals. The central or peripheral administration of AMPK activators in TB rats caused an increase in food intake that was apparent 4 hours after treatment and lasted for 12 and 24 hours (Fig. 3A and B). When criteria for anorexia had been met, we administered a daily injection of icv AICAR or ip 2DG during 4 days in TB rats to evaluate the cumulative food intake. In TB animals the cumulative food intake was reduced by about 75% when compared to respective control groups; however, the chronic treatment with AICAR (i.c.v.) or 2DG (i.p.) increased the cumulative food intake by about 25 and 35%, respectively, when compared to TB rats treated with i.c.v. or i.p. saline (Fig. 3C and D).

In addition, we investigated whether the hypothalamic AMPK activation increases survival in TB animals after chronic AICAR or 2DG treatment. As shown in the Kaplan-Maier's graphs, a daily central infusion of AICAR or a peripheral injection of 2DG, statistically prolonged the survival in TB animals, whereas, the median survival of TB rats after chronic administration of saline via i.c.v. or i.p. was ~5.5 days and chronic administration of AICAR or 2DG increased the median survival to 9.5 and 8.0 days, respectively (Fig. 3E and F).

Hypothalamic AMPK activation decreases the expression of proinflammatory molecules in TB rats

We next investigated the anti-inflammatory properties of AICAR in the hypothalamus of TB rats. To test the hypothesis that AMPK activation reduces the production of proinflammatory cytokines in TB rats, we injected the AMPK activator and evaluated the hypothalamic expression of iNOS, IL-1 β and TNF- α . The protein levels of iNOS increased by 3.8-fold in the hypothalamus of TB rats when compared to control animals, and the micro infusion of AICAR reduced iNOS expression by 1.5-fold in the hypothalamus of TB rats, when compared to saline-treated TB rats (Fig. 4A). In addition, the protein levels of IL-1 β and TNF- α were increased by 7.9 and 2.0-fold, respectively, in the hypothalamus of TB rats when compared to the control group, and AMPK activation reduced the hypothalamic expression of IL-1 β by 1.9-fold and TNF- α by 1.5-fold, when compared to TB rats treated with i.c.v. saline (Fig. 4B and C, respectively).

Central infusion of AICAR increased NPY and reduced POMC mRNA levels in the hypothalamus of TB rats.

To explore the mechanism(s) by which AMPK activation regulates food intake in TB rats, we examined the expression of hypothalamic neuropeptides involved in the control of energy homeostasis in TB animals. After 12 hours of fasting, the neuropeptide Y (NPY) mRNA levels were reduced by 3.1-fold in the hypothalamus of TB rats when compared to the control group, and the i.c.v. microinfusion of AICAR increased hypothalamic NPY mRNA levels by 2.0-fold in TB rats when compared to saline-treated TB rats (Fig. 5A). Consistent with these findings, pro-opiomelanocortin (POMC) mRNA levels were increased by 3.1-fold in the hypothalamus of TB rats when compared to the control group, and the i.c.v. microinfusion of AICAR decreased hypothalamic POMC mRNA levels by 2.1-fold in TB rats when compared to saline-treated TB rats (Fig. 5B).

Oral administration of metformin induced hypothalamic AMPK activation, attenuates cancer anorexia and prolonged survival in tumor bearing rats.

To determine whether the anti diabetic drug, metformin, induces hypothalamic AMPK phosphorylation and increases food intake and survival in TB rats, we chronically administered metformin using oral gavage during cancer-induced anorexia in rats. The oral administration of metformin (500 mg/kg) in TB rats caused an increase in hypothalamic AMPK phosphorylation in a time-manner dependent, with maximal response at 180 minutes after oral gavage (Fig. 6A-upper panel). The total AMPK protein levels were not different between the groups after metformin administration (Fig. 6A-lower panel).

Next, we explored the effects of oral gavage of metformin on hypothalamic AMPK and ACC phosphorylation in the TB animal. Under fasting conditions, AMPK and ACC phosphorylation were reduced by 3.1- and 4.6-fold in the hypothalamus of TB rats when compared to the control group, respectively. Metformin administration increased hypothalamic AMPK and ACC phosphorylation by 2.0- and 3.9-fold, when compared to saline-treated TB rats (Fig. 6 B and C-upper panels). The AMPK and ACC protein levels were not different between the groups after metformin administration, respectively (Fig. 6 B and C-lower panels).

Figure 6 D shows that the tumor development markedly reduced food intake during 24 hours in TB rats after saline treatment when compared to control animals. Furthermore, oral administration of metformin in TB rats, caused an increase in food intake that was apparent 4 hours after treatment and lasted for 12 and 24 hours (Fig. 6D). When criteria for anorexia had been met, the cumulative food intake was evaluated after a daily oral gavage treatment with saline or metformin during 4 days in TB animals. The cumulative food intake was reduced by about 75% when compared to control animals, however, the daily treatment with metformin, increased the cumulative food intake by about 28%, when compared to TB rats treated with oral saline (Fig. 6E).

Finally, we investigated whether the hypothalamic AMPK activation increases the survival in TB animals after chronic oral treatment with metformin. Figure 6F shows, a daily oral administration of metformin significantly increases survival, whereas the median survival of oral saline-treated TB rats was 5.5 days and chronic administration of oral metformin increased the median survival to 8.5 days.

DISCUSSION

In the present study, we demonstrate that central AMPK activation can attenuate cancer-induced anorexia, whereas, the activation of neuronal AMPK inhibits the hypothalamic expression of proinflammatory molecules, increases the NPY and suppresses POMC mRNA levels in the hypothalamus of anorectic tumor bearing rats. Interestingly, different pharmacological activators of AMPK signaling, including intracerebroventricular microinfusion of AICAR, intraperitoneal injection of 2DG and oral administration of metformin, activate AMPK in the central nervous system, increase food consumption and prolong life span in the model of cancer-induced anorexia.

An increased expression of cytokines during tumour growth prevents the hypothalamus from responding appropriately to peripheral signals by persistently activating anorexigenic systems and inhibiting prophagic pathways (3). Convincing evidence suggests that cytokines may cross the blood brain barrier and have a vital role, triggering the

complex neurochemical cascade, leading to the onset of cancer anorexia (3, 5). In addition, cytokine production in brain regions distant from the brain tumor site may also be involved in this clinical manifestation (28). In patients with cancer, it is probable that cytokines and anorexia are connected, since the biological effects of cytokines are largely mediated by paracrine and autocrine influences (3). In the Fischer rat/MCA sarcoma model, brain IL-1 β concentrations inversely correlate with food intake (29) and intrahypothalamic microinjections of an interleukin-1-receptor antagonist increases energy intake (30). Tumor necrosis factor- α (TNF- α) is one of the mediators of the hypothalamic anorexigenic signals that participate in the induction of the cachexia syndromes present in advanced stage cancer and in severe infectious (31, 32). Our data demonstrated that the reversal effect of AMPK on cancer induced-anorexia is associated with decreased concentrations of iNOS, IL-1 β and TNF- α in the hypothalamus of tumor-bearing rats. The anti-inflammatory response produced by AMPK activation in the CNS is in accordance with a several studies, suggesting that demonstrate that AICAR inhibited production of proinflammatory mediators (TNF- α , IFN γ , and NO) in brain glial cells, primary astrocytes, microglia, and peritoneal macrophages (20, 22). In addition, the pharmacological activation of AMPK also inhibited the production of proinflammatory molecules in serum and their expression in the CNS of rats injected with a sublethal dose of LPS by intraperitoneal injection (20).

The mechanisms involved in the proinflammatory cytokine-dependent modulation of food intake and energy expenditure may also involve the control of hypothalamic neurotransmitter expression (33, 34), in situ stimulation of the expression of other cytokines, particularly IL-1 β (35, 36), and the activation of anorexigenic leptin-like signal transduction in the hypothalamus (37). IL-1 β has been clearly associated with the induction of anorexia (32), by blocking neuropeptide Y (NPY)-induced feeding. The levels of this molecule are reduced in anorectic tumor-bearing rats (38), and a correlation between food intake and brain IL-1 has been found in anorectic rats with cancer. The mechanism involved in the attenuation of NPY activity by cytokines may be related to an inhibition of cell firing rates or to an inhibition of NPY synthesis or an attenuation of its postsynaptic effects (10). On the other hand, it has been demonstrated that POMC-containing cells contain cytokine receptors, and that these neurons are activated during acute and chronic

inflammation (8). This discovery demonstrates that proinflammatory cytokines such as TNF- α , IL-1 β and IL-6 can bind to cytokine receptors on POMC-containing cells, causing these cells to increase their signaling to second-order neurons that affect outputs related to anorexia, loss of lean body mass and increased energy expenditure. In our model of cancer-induced anorexia, we observed high levels of iNOS, IL-1 β and TNF- α in the hypothalamus of Walker tumor-bearing rats and, after 12 hours of fasting, the mRNA levels of NPY were lower and POMC were much higher than in control animals. Notably, we also observed defective 172 threonine AMPK phosphorylation in the hypothalamus of anorexic tumor-bearing rats under fasting conditions, when compared to normal rats, suggesting that the neuronal AMPK could be involved in cancer-mediated anorexia.

AMPK is expressed in the hypothalamic neurons involved in the regulation of food intake (39), whereas, the central pharmacological activation of AMPK by AICAR leads to increased food intake and decreased energy expenditure in normal mice (15). Furthermore, acute intraperitoneal administration of 2DG increased food intake and both the expression of AgRP and NPY in the arcuate nucleus (40). These findings were further explored in different studies, demonstrating that increasing hypothalamic AMPK activity increased the expression of orexigen neuropeptides in the arcuate nucleus of the hypothalamus, leading to increased food intake (41, 42). We demonstrate that the microinfusion of AICAR activates AMPK in neuronal cells in a time- and dose-dependent manner and that the higher dose of AICAR (2mM) partially restores the NPY and POMC mRNA levels in the hypothalamus of anorectic rats under fasting conditions. Moreover, both central microinfusion of AICAR and intraperitoneal injection of 2DG increased food intake and prolonged survival in tumor-bearing animals.

AMPK provides a candidate target, capable of mediating the beneficial metabolic effects of metformin (43). Metformin lowers blood glucose and blood lipid contents, and these effects are thought to be at least partially responsible for its therapeutic benefits (44, 45). Metformin decreases the leptin concentration in morbidly obese subjects (46, 47) and in normal-weight healthy men (48). Metformin has been suggested to act through the stimulation of AMPK in peripheral tissues; however, few studies have demonstrated whether metformin modulates AMPK activity in the neuronal cell. In Zucker

rats, a single subcutaneous dose of metformin (300 mg/kg) reduced food intake only in obese animals, while the same dose of metformin given orally did not affect food intake in either lean or obese animals (49). In an acute study, metformin treatment increased the anorexic effect of leptin, and this was accompanied by increased levels of phosphorylated signal transducer and activator of transcription 3 (STAT3) in the hypothalamus of high-fat-fed obese rats (50). On the other hand, our results indicated that a high dose of metformin (500 mg/kg) increases the phosphorylation of the AMPK/ACC pathway in a time-dependent manner in the hypothalamus of rodents in cancer-induced anorexia under fasting conditions. A daily oral administration of metformin blockaded the anorectic response, leading to increased food intake and life span in tumor-bearing rats. These apparent contradictory effects of metformin in the hypothalamus may be related to the different physiological and metabolic profiles observed in these studies. In addition, we used a prolonged fasting period to evaluate the hypothalamic AMPK phosphorylation. Furthermore, we performed the same experiments with an oral administration of metformin (500 mg/kg) in normal rats and we did not observe changes in either food intake or body weight, suggesting that the physiological and metabolic parameters are essential to metformin-induced changes in food intake and body weight in rodents.

In summary, the findings of this study suggest that neuronal AMPK activation reverses cancer anorexia by inhibiting the production of proinflammatory molecules and controlling the expression of neuropeptides involved in the control of food intake, reflecting in the prolonged life span of tumor-bearing rats. Thus, our data indicate that hypothalamic AMPK activation presents an attractive opportunity for the treatment of cancer-induced anorexia, whereas, the restoration of appetite in cancer patients is likely to improve quality of life and might also improve overall patient survival.

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FIGURE LEGENDS

Figure 1- Schematic representation of the experimental procedures. Surgical implantation of ICV cannulae was performed ~3 weeks before the in vivo study. Full recovery of body weight and food intake was achieved after 7 days. When criteria for anorexia had been met, ICV infusions were started and blood chemistries, food intake and/or survival analysis were assessed.

Figure 2- The basal levels of hypothalamic AMPK phosphorylation and the time-course and dose-response after central infusion of AICAR or peripheral administration of 2DG in tumor-bearing rats. Representative Western blots demonstrating: (A) The effect of 12 hours fasting on hypothalamic AMPK phosphorylation in control and tumor-bearing animals. (B) The hypothalamic AMPK phosphorylation in a time- and dose-dependent manner after central infusions of AICAR, and after intraperitoneal injections of 2DG in tumor-bearing rats (C). Tissue extracts were immunoblotted (IB) with anti- phospho AMPK antibody (upper panels) and anti- AMPK α antibody (lower panels). The results of scanning densitometry were expressed as arbitrary units. Bars represent means $\pm$ SEM of six to eight rats. # $p < 0.05$, vs. control fasted and * $p < 0.05$, vs. respective vehicles.

Figure 3- The effects of intracerebroventricular infusion of AICAR or intraperitoneal 2DG on food intake and on survival in tumor-bearing (TB) rats. Determination of 24- hours of food intake after AICAR microinfusion (A) or after 2DG injection (B). Determination of 4 days of cumulative food intake after AICAR (C) or 2DG treatment (D). Results represent means $\pm$ SEM of six to eight rats. # $p < 0.05$, vs. saline and * $p < 0.05$, vs. TB rats + saline. Representative survival curves demonstrating the effect of chronic administration of AICAR (E) or 2DG (F) on life span of tumor-bearing animals. The survival curves were estimated using Kaplan-Meier's estimates, and curves were compared using the log-rank test, $p < 0.001$ (n=10).

Figure 4- Intracerebroventricular infusion of AICAR attenuated the production of proinflammatory molecules in the hypothalamus of tumor-bearing rats. Representative Western blots demonstrating the effects of AICAR on iNOS (A) production in the hypothalamus of tumor bearing rats. Bars represent means $\pm$ SEM of six rats. Tissue

extracts were immunoblotted (IB) with anti – iNOS antibody. ELISA assay demonstrated the anti- inflammatory proprieties of AICAR on IL-1 β (B), and TNF- α (C) expression in the hypothalamus of tumor-bearing rats (n =5 per group). #p<0.05, vs. saline and *p<0.05, vs. TB rats + saline.

Figure 5- The effect of central infusion of AICAR on NPY and POMC mRNA levels in the hypothalamus of tumor bearing rats in fasting conditions. The RT-PCR demonstrated the NPY (A) and POMC (B) mRNA levels after AICAR microinfusion. The results of scanning densitometry were expressed as arbitrary units. Bars represent means $\pm$ SEM of six rats. #p<0.05, vs. saline and *p<0.05, vs. TB rats + saline.

Figure 6- Oral administration of metformin activated hypothalamic AMPK, attenuated cancer-induced anorexia and prolonged survival in tumor-bearing rats. Representative Western blots demonstrating the effect of oral metformin on hypothalamic AMPK phosphorylation in a time-dependent manner (A). Representative Western blots demonstrating the effect of oral metformin on hypothalamic AMPK and ACC phosphorylation (B and C). Tissue extracts were immunoblotted (IB) with anti- phospho AMPK antibody and anti- phospho ACC (upper panels) and anti- AMPK α and anti- ACC antibodies (lower pannels). The results of scanning densitometry were expressed as arbitrary units. Bars represent means $\pm$ SEM of six rats. #p<0.05, vs. saline and *p<0.05, vs. TB + saline. Determination of 24-food intake after oral metformin administration (D). Determination of 4 days of cumulative food intake after oral metformin administration (E). Effect of chronic administration of oral metformin on life span (F). The survival curve was estimated using Kaplan-Meier's estimates, and curves were compared using the log-rank test, p <0.001 (n=10).

TABLE 1. Characteristics of tumor bearing (TB) rats and their age-matched controls.

Groups (n=6)	Control	TB+Saline	TB+AICAR	TB+2DG	TB+Metformin
Body weight (g)	369.3±5.3	346.5±7.6*	355.5±3.7* #	361.6±7.0* #	356.3±3.3* #
Body weight excluding tumor (g)	369±5.3	310.9±12.2*	314.3±5.5* #	329.2±9.1* #	318.4±6.3* #
Tumor weight (g)	—	35.6±3.6	41.3±2.3	32.4±2.7	35.0±2.5
Fasting glucose (mg dl⁻¹)	80.2±2.6	85.4±4.0	81.8±4.9	81.7±7.8	85.5±2.8
Fasting insulin (ng ml⁻¹)	1.05±0.17	0.06±0.01*	0.41±0.1* #	0.16±0.01* #	0.64±0.17* #
Fasting leptin (ng ml⁻¹)	5.62±1.4	0.21±0.04*	0.39±0.03* #	0.48±0.06*	1.11±0.05* #
Kitt (% min⁻¹)	4.53±0.6	1.93±0.4*	2.84±0.5* #	2.54±0.3* #	3.32±0.4* #

* $p < 0.05$, vs. control group.

$p < 0.05$, vs. TB+saline group.

Figure 1

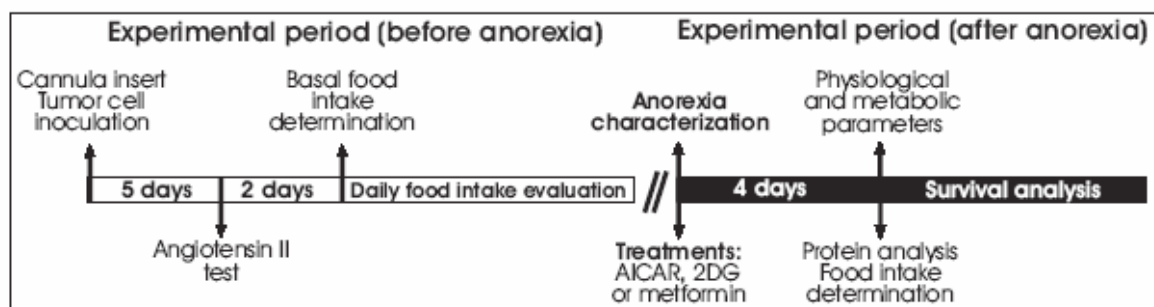


Figure 2

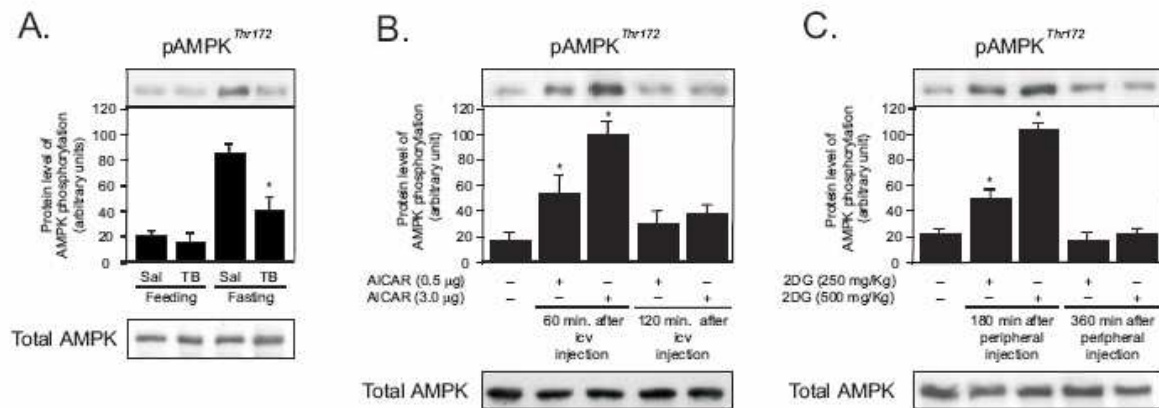


Figure 3

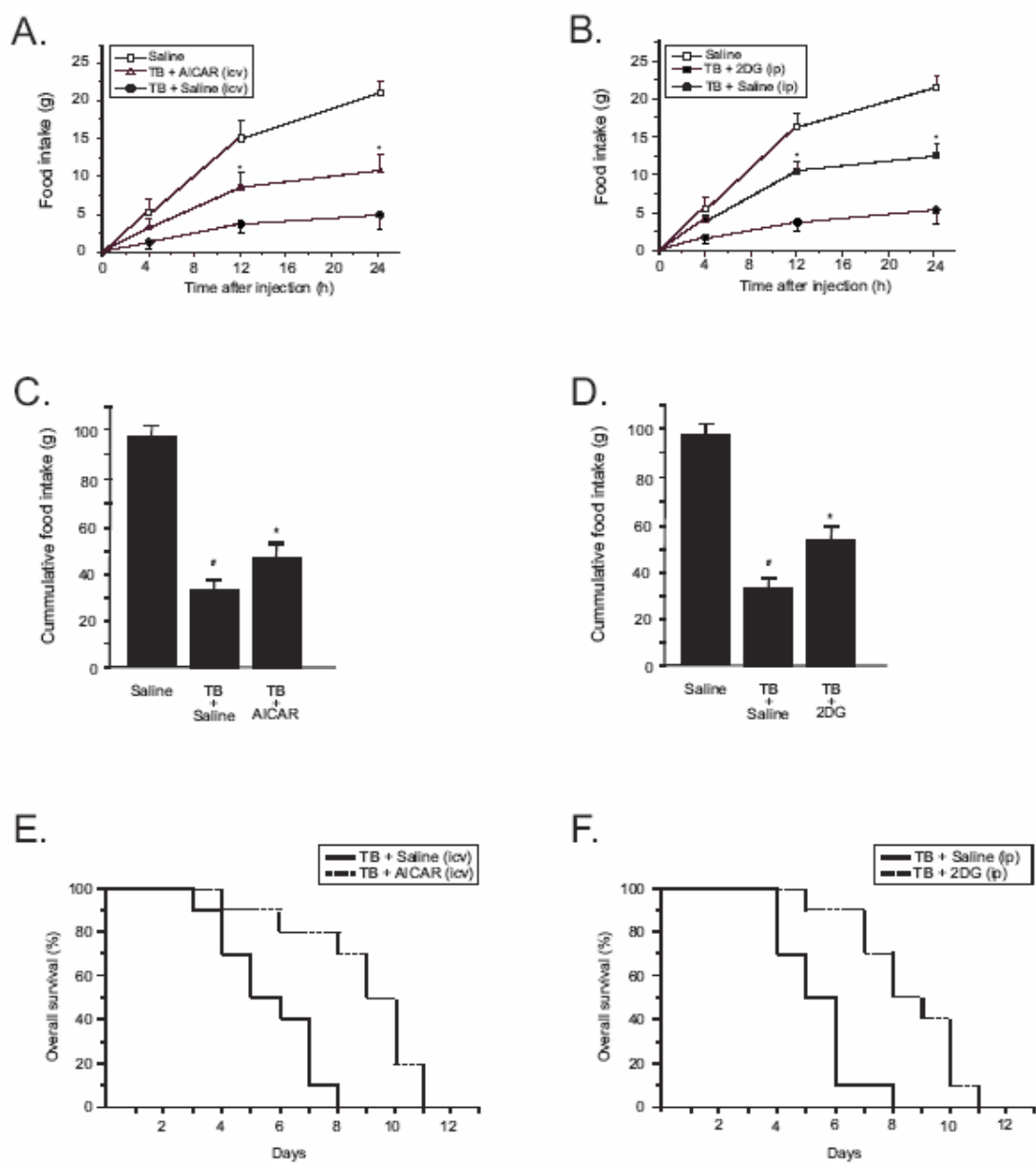


Figure 4

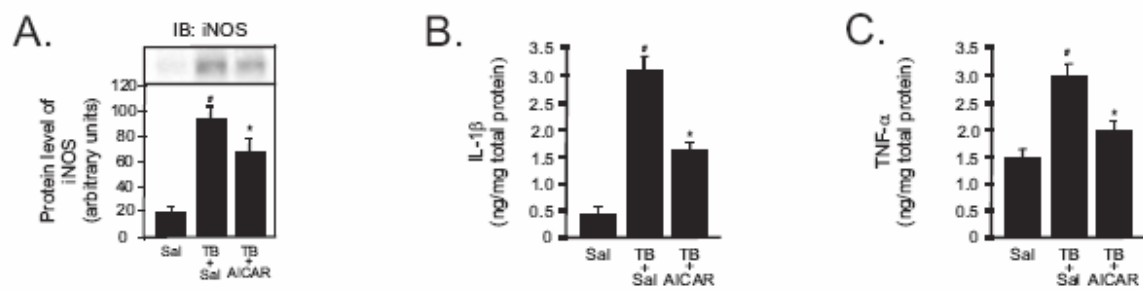


Figure 5

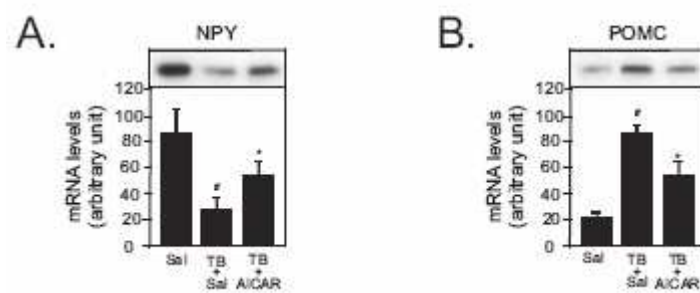
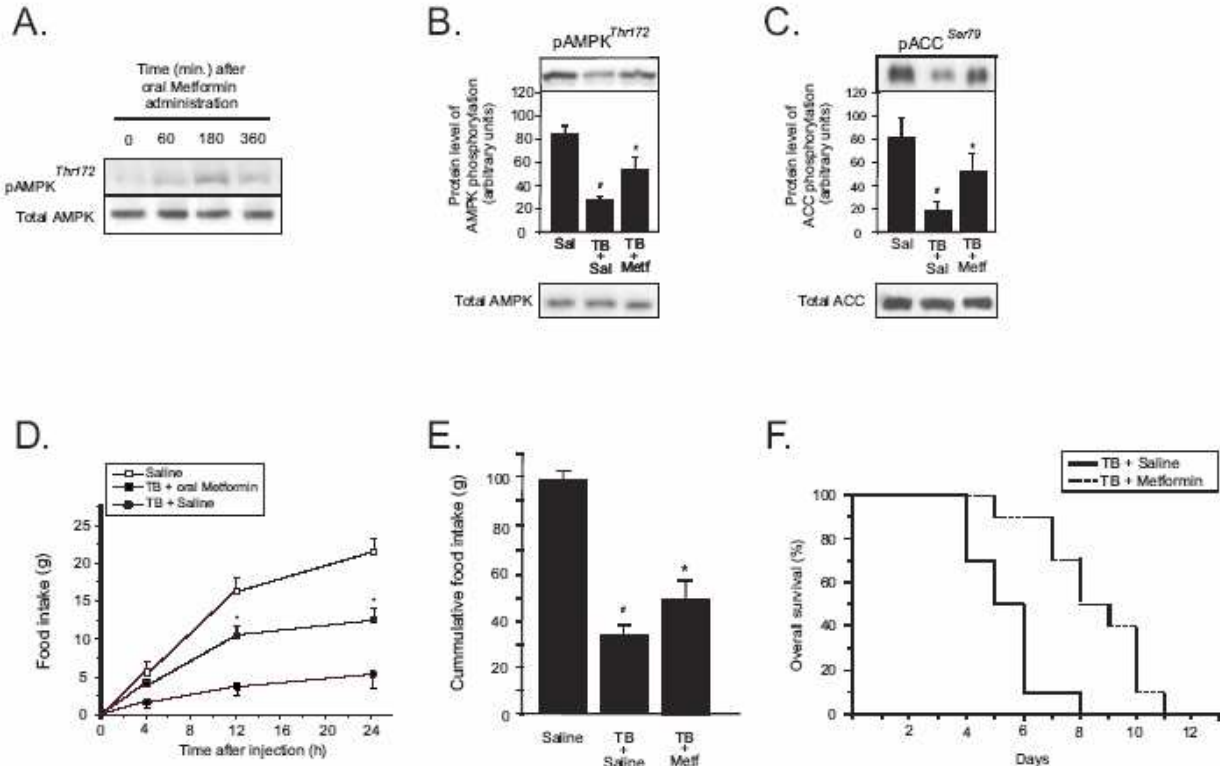


Figure 6



4- CONCLUSÃO

O presente estudo caracterizou a sinalização da AMPK no hipotálamo de ratos com anorexia induzida pelo desenvolvimento tumoral e documentou que a administração de diferentes farmacoss ativadores da AMPK como o AICAR e 2DG, foram capazes de restabelecer parcialmente a atividade da AMPK hipotalâmica, e reverter a anorexia induzida pelo câncer. Nossos dados desmonstram que a ativação da AMPK reduz a expressão de moléculas pró-inflamatórias (iNOS, IL-1 β e TNF- α no SNC e modula a expressão dos neuropeptídeos, esses efeitos foram responsáveis pelo aumento da ingestão alimentar e da sobrevida dos animais portadores de tumor. O estudo demonstrou também que o uso oral de metformina também foi capaz de ativar a via AMPK/ACC no hipotálamo e aumentar a ingestão alimentar e a sobrevida. Assim, estes dados sugerem que a ativação da AMPK neuronal representa um potencial alvo terapêutico para o tratamento da anorexia em pacientes com câncer.

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