Luiz Fernando de Rezende

Efeitos do Ciliary Neurotrophic Factor (CNTF) sobre a função e sobrevivência de Ilhotas pancreáticas

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Tese apresentada no Instituto de Biologia para obtenção de título de Doutor em Biologia Funcional e Molecular na área de Fisiologia.

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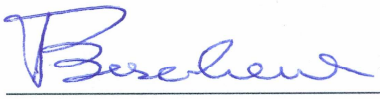

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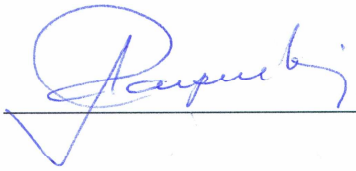

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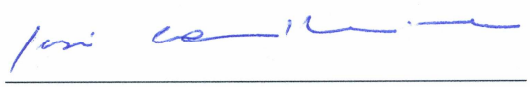

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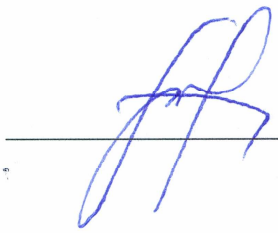

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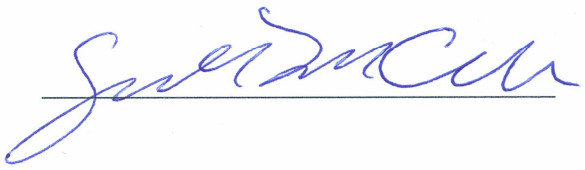

Dedico este trabalho à minha esposa Rosângela e a todos que de alguma forma contribuíram com a realização dele.

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SUMÁRIO

LISTA DE ABREVIATURAS

ADP – Adenosine Di-Phosphate

AG490 - Tyrphostin B42 *N*-Benzyl-3,4-dihydroxy-benzylidenecyanoacetamide

AKT – PKB, Protein Kinase B

ATP – Adenosine Tri-Phosphate

BAD – Bcl-2-associated death promoter

BAX – Bcl-2–associated X protein

BCL-2 – B-cell lymphoma 2

BSA – Bovine Serum Albumin

Chaps – 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CNTF – Ciliary Neurotrophic Factor

CNTFRα – Ciliary Neurotrophic Factor Receptor alpha

Cx36 – Connexin 36

DEVD – Amino acid sequence: Asp-Glu-Val-Asp

DM1 – Diabetes Mellitus Tipo 1

DM2 – Diabetes Mellitus Tipo 2

DTT – Dithiothreitol

ERK – Extracellular signal-regulated kinases

FBS – Fetal Bovine Serum

GCK4 – Glucokinase 4

GLUT2 – Glucose Transporter 2

Gp130 – Glicoprotein 130

GSIS – Glucose-Stimulated Insulin Secretion

Hepes – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IDDM – Insulin Dependant Diabetes Mellitus

IL-1β – Interleukin 1 beta

IL-6 – Interleukin 6

IL-11 – Interleukin 11

INFγ – Interferon gama

JAK – Janus Kinase

K_{ATP} – ATP-sensitive potassium (K_{ATP}) channels

MAPK - Mytogen Activated Protein Kinase

mRNA – Messenger Ribonucleic Acid

MPT – Mitochondrial permeability transition

MTS – 3-[4, 5, dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4- sulfophenyl]-2H-tetrazolium

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NKX6.1 – NK6 homeobox 1

NO – Nitric Oxide

NGFs – Nerve Growth Factors

PAX4 – Paired Box Gene 4

PD98059 – 2-(2-amino-3-methoxyphenyl)-4h-1-Benzopiran-4-one

PDX-1 – Pancreatic and duodenal homeobox 1

PI3K – Phosphatidylinositol-3-OH kinase

PKC – Protein Kinase C

PMS – phenazine methosulfate

pNA – Paranitroanilide

RIA – Radioimunoassay

ROS – Reactive Oxigen Species

RPMI – Roswell Park Memorial Institute

RPS-29 – Ribosomal protein S29

RT-PCR – Reverse Transcriptase-Polymerase Chain Reaction

SDS-PAGE – Sodium Sodecyl Sulfate PolyAcrylamide Gel Glectrophoresis,

SNC – Sistema Nervoso Central

SNP – Sistema Nervoso Periférico

SOCS-3 – Supressor of Cytokine Signaling 3

STAMs – Signal-transducing adapter molecules

STAT – Signal Transducers and Activators of Transcription

StIP – Stat Interacting proteins

TBE – Tris Borate EDTA

TBS – Tris-Buffered Saline

TNFα – Tumor necrosis factor alpha

VDAC – Voltage- Dependant Anion Channels

RESUMO

Introdução: O CNTF pertence à família da IL-6, e sendo assim, sinaliza pelo complexo receptor gp130, ativando diversas vias de sinalização dependendo do tipo celular, principalmente as vias STAT3, MAPK e PI3K. Seus efeitos incluem diferenciação e/ou sobrevivência neuronal, e é diferencialmente expresso ao longo da vida do animal. As ilhotas pancreáticas, por sua vez, são ricamente enervadas e expressam receptores para NGFs, podem apresentar respostas neurotípicas e expressam o CNTF. O objetivo desse estudo foi investigar os possíveis efeitos do CNTF sobre a diferenciação e/ou sobrevivência de ilhotas pancreáticas de ratos neonatos, qual(is) via(s) de sinalização ele ativa nessas ilhotas e como é expresso nelas ao longo da vida dos animais.

Material e Métodos: Ilhotas pancreáticas de ratos neonatos (1-2 dias) foram isoladas pelo método de colagenase e cultivadas por 3 dias em meio RPMI com (CNTF) ou sem (CTL) 1nM de CNTF. Após isso, foram analisados a secreção de insulina estimulada por glicose (RIE), metabolismo (MTS, produção de NADPH), metabolismo de glicose (produção de $^{14}CO_2$), expressão gênica (RT-PCR), protéica (Western-Blot), atividade de caspase-3 (DEVD) e apoptose (fragmentação de DNA).

Resultados: O CNTF reduziu a secreção de insulina estimulada por glicose e o metabolismo de ilhotas pancreáticas, não alterando o metabolismo de glicose e expressão de proteínas cruciais para a função das ilhotas. Por outro lado, o CNTF aumentou a expressão de proteínas relacionadas à sobrevivência das ilhotas pancreáticas, como Cx36, PAX4, e BCL-2, reduziu a atividade da caspase-3 e a apoptose das ilhotas. O CNTF também aumentou a fosforilação de STAT3, sua translocação ao núcleo e expressão de genes-alvo, como a SOCS-3, levando à redução da GSIS e sobrevivências observadas, apesar de não ativar as vias da MAPK e PI3K. Mais ainda, a expressão do CNTF é aumentada em ilhotas pancreáticas de ratos de 2 meses de idade, e assim permanecendo até os 20 meses de idade.

Conclusão: O CNTF não promove a maturação de ilhotas pancreáticas, mas sim sua sobrevivência, e esses efeitos são mediados através da via JAK/STAT3, sem ativar as vias MAPK ou PI3K. Finalmente, o CNTF possui expressão diferenciada ao longo da vida do animal.

ABSTRACT

Introduction: CNTF belongs to the IL-6 cytokine family and as such, it signals through gp130 receptor complex, activating many pathways depending on the cell-type, mainly STAT3, MAPK and PI3K. Its effects include increased neuron differentiation and/or survival, and are differentially expressed throughout the animal life. Meanwhile, pancreatic islets are richly innervated and express receptors for NGFs, may undergo neurotypic responses, and express CNTF. The aim of this study was to investigate possible effects of CNTF on neonatal rat pancreatic islet differentiation and/or survival, which signalling pathway (s) it activates on pancreatic islets and how it is expressed in the pancreatic islets throughout the animal life.

Methods: Pancreatic islets from neonatal rats (1-2 d old) were isolated by the collagenase method and cultured for 3 days in RPMI medium with (CNTF) or without (CTL) 1nM of CNTF. Thereafter, glucose-stimulated insulin secretion (RIA), general metabolism by (NAD(P)H production) (MTS), glucose metabolism ($^{14}CO_2$ production), gene (RT-PCR), protein expression (Western-Blot), caspase-3 activity (DEVD), and apoptosis (DNA fragmentation) were analysed.

Results: CNTF reduced pancreatic islets GSIS and metabolism, whereas not affecting glucose metabolism and the expression of proteins crucial for the islets function. Conversely, CNTF significantly expression of proteins related pancreatic islets survival, such as Cx36, PAX4, and BCL-2, reduced caspase-3 activity and islet-cells apoptosis. CNTF also increases STAT3 fosforilation, translocation to the nuclei and expression of target genes, resulting in the reduced GSIS and survival observed, although not affecting MAPK and PI3K pathways. Moreover, CNTF expression is increased in rats pancreatic islets after 2 months of age, and it remains so until 20 months of age.

Conclusion: CNTF has no effect over maturation of pancreatic islets function, whereas it improves pancreatic islets survival, and also that these effects are mediated through JAK/STAT3 but not through MAPK or PI3K pathways. Finally, CNTF is differentially expressed in rat pancreatic islets throughout the animal life.

INTRODUÇÃO

Descrição do Diabetes

A secreção de insulina pelas células-β pancreáticas é ajustada continuamente de acordo com as flutuações dos níveis de nutrientes circulantes, e, em especial, da glicose. A secreção é modulada ainda, direta ou indiretamente, por neurônios, neurotransmissores e agentes farmacológicos. Esse controle multifatorial permite que as células-β secretem insulina em quantidade e tempo adequados, regulando perfeitamente os níveis de nutrientes no sangue em diversas condições fisiológicas, tais como: jejum, refeição, exercício físico, gravidez, lactação, crescimento [1].

A insulina é o mais importante anabolizante em mamíferos, sendo fundamental para a manutenção da homeostasia da glicose, ácidos graxos e aminoácidos. Devido à crescente ocorrência de diabetes mellitus, sobretudo nos países desenvolvidos, esse hormônio tem sido pesquisado intensamente [2].

A ausência da insulina, característica de Diabetes Mellitus Tipo 1 (DM1) ou secreção diminuída da mesma pela incapacidade das células-β em metabolizar adequadamente glicose (diabetes Moddy) ou, ainda, secreção aparentemente normal mas insuficiente para suplantar a resistência à insulina nos tecidos alvo, como no Diabetes Mellitus Tipo 2 (DM2) leva ao descontrole da homeostasia de carboidratos e de triglicérides. A síntese protéica bem como o controle nervoso central (SNC) da ingesta alimentar [3] e do tônus vascular periférico [4] também ficam alterados. Ainda, ocorre acúmulo de nutrientes no plasma e dificuldades no processamento intracelular dos mesmos, levando à redução das funções fisiológicas e posterior degeneração de sistemas e órgãos [5].

No DM1 o envolvimento do estresse oxidativo com a destruição das células-β é de forma aguda e agressiva: há falha no reconhecimento de proteínas específicas das células-β pelo sistema imune e infiltração das ilhotas por neutrófilos ativados, que liberam H_2O_2 - produzindo concentrações localmente elevadas (em torno de 1 mM) desse oxidante – além de citocinas como INFγ [6], TNFα [7] e IL-1β [8]. As células-β possuem receptores para estas citocinas, que ativam vias apoptóticas, de supressão da síntese protéica e expressão de Fas na membrana plasmática, um marcador para a

ligação de linfócitos T. Após semanas ou meses (parâmetros humanos), há perda quase total das células-β, sem prejuízo dos demais tipos celulares que compõem a ilhota pancreática. No DM2, a síndrome se instala pela dificuldade que as células-β apresentam em secretar concentrações crescentes de insulina para fazer frente ao aumento da resistência periférica á insulina.

O CNTF

O Ciliary Neurotrophic Factor (CNTF), a exemplo da IL-6, IL-11, Leukemia Inhibitory Factor, Cardiotrophin-1 e Oncostatin M, pertence à família da interleucina–6 (IL-6). O CNTF foi primeiramente reconhecido como um fator de sobrevivência neuronal, mais precisamente, de sobrevivência de neurônios ciliares parassimpáticos, além de outros efeitos neurotróficos tais como: fator de sobrevivência de neurônios simpáticos, sensoriais e motores *in vitro* e *in vivo*, [9, 10], e fator de diferenciação de células progenitoras de astrócitos em astrócitos tipo 2 [11, 12].

O CNTF é encontrado no Sistema Nervoso Central (SNC) primariamente em neurônios e células da Glia, enquanto no Sistema Nervoso Periférico (SNP) ele é encontrado em altas concentrações nas células de Schwann. Como não possui domínio sinalizador para exocitose [12], sugeriu-se que o CNTF é uma proteína citossólica, liberada no meio extracelular como conseqüência de danos celulares.

Alterações na distribuição e expressão de mRNA e proteína de CNTFRα durante o desenvolvimento da retina de ratos têm sido descritas [13, 14], e é conhecida a importância do CNTF para a sobrevivência neuronal e diferenciação celular no sistema nervoso [12]. Além disso, o CNTF tem efeitos antagônicos podendo ser catabólico ou anabólico, dependendo do tecido e do estágio da vida [15]. A injeção intravenosa de CNTF de até 1 nM induz hipoglicemia, potencializa a produção de IL-6 e aumenta a secreção de corticosterona induzida por IL-1 [16].

Interação entre o CNTF e as ilhotas pancreáticas

Assim como todos os outros membros da família das IL-6, o CNTF usa a via da glicoproteína gp130 como elemento de transdução de sinais [17]. O complexo receptor funcional de CNTF (CNTF-R) consiste da gp130, do Leukemy Inhibitory Factor Receptor e do receptor específico de CNTF (CNTF-Rα). Este não possui domínio citoplasmático e permanece ancorado à membrana via ligação a glicosil-phosphatidil-inositóis. É relatado que a IL-6 inibe a secreção de insulina induzida por glicose em ilhotas de ratos [18, 19], além de potencializar o efeito (inibitória) da IL-1β sobre a secreção de insulina [19]. Além disso, células-β expressam receptores para diversos fatores de crescimento nervosos (NGFs) e podem ter uma resposta neurotípica a diversos fatores de diferenciação neuronal [20]. As ilhotas de Langerhans são ricamente inervadas e o CNTF, presente nas células de Schwann do SNP [11], pode ter função parácrina uma vez que interfere também em células não-neuronais [21].

Entre os efeitos do CNTF sobre o controle glicêmico de animais tratados com o polipeptídeo destacam-se: o menor peso corporal e menor adiposidade em ratos db/db (deficientes em leptina) e redução da hiperinsulinemia e hiperlipidemia associadas. Esses efeitos do CNTF foram atribuídos à menor ingestão de alimentos associado ao aumento da taxa metabólica basal desses animais [22]. Apesar de reduzir a secreção de insulina estimulada por glicose [23], o CNTF é apontado como antidiabetogênico em modelos de resistência à insulina e/ou leptina e DM2 em ratos, exibindo diversos efeitos sistêmicos in-vivo, como redução da adiposidade, peso corporal, hiperinsulinemia e hiperglicemia. [22, 24, 25, 26, 27].

Algumas evidências da ação do CNTF sobre as ilhotas pancreáticas têm sido relatadas.

O CNTF potencializou o efeito inibitório de IL-1β sobre a secreção de insulina bem como a síntese de NO estimulada por IL-1β em ilhotas de ratos. Não se conhece os mecanismos envolvidos

na mediação desses efeitos. Provavelmente, estão associados à via da gp130, uma vez que os mesmos são mimetizados pela administração da IL-6 [23].

Apesar de ser conhecida a via de sinalização do CNTF em células neuronais [28, 29] e existir evidências de que essa via em ilhotas pode ser semelhante, os resultados são ainda inconclusivos e os mecanismos de ação do CNTF em células-β pancreáticas permanecem desconhecidos.

Sinalização do CNTF via STAT3

É conhecido que, na maioria dos tipos celulares, O CNTF exerce seus efeitos através da via de sinalização JAK/STAT3 [22, 29, 30]. O CNTF se liga ao seu receptor CNTFR presente no complexo gp130, ativando JAK2 [31] e fosforilando resíduos de tirosina no CNTFR, recrutando e fosforilando a STAT3, que por sua vez se dimeriza e é translocada ao núcleo, onde regula a transcrição gênica [29, 30]. Porém, o CNTF também pode sinalizar através das vias PI3K/AKT [32, 33] e/ou MAPK/ERK [34], ambas podendo ocorrer de forma concomitante ou independente da sinalização via STAT3.

Ativação de vias JAK/STATs em geral estimula proliferação, diferenciação, migração e sobrevivência celular, e a via da STAT3 não é uma exceção, visto ter sido descrita como uma via de sobrevivência, anti-apoptótica e antiinflamatória [35, 36].

OBJETIVOS

O CNTF exerce tanto efeitos de diferenciação/maturação quanto de sobrevivência, dependendo do tipo celular estudado, por isso, decidimos avaliar qual seu efeito sobre as ilhotas pancreáticas. Ainda, o CNTF pode exercer sua atividade através de diferentes vias de sinalização, como JAK/STAT3, PI3K/AKT ou MAPK/ERK, e seria de grande importância a elucidação de qual(is) a(s) via(s) que ele utiliza para sua ação sobre ilhotas pancreáticas. Por fim, a expressão do CNTF e de seu receptor é alterada ao longo da vida do animal, o que se está relacionado a alterações fisiológicas. Os objetivos do presente trabalho podem ser assim explicitados:

1-) Verificar se o CNTF exerce efeitos de diferenciação/maturação sobre as ilhotas pancreáticas através da análise da expressão gênica de proteínas determinantes de células-β como PDX, CX36, PKC, GLUT2, GCK4 e NKX 6.1.

2-) Verificar se o CNTF promove sobrevivência de ilhotas pancreáticas através da análise de apoptose por fragmentação de DNA e atividade de Caspase 3, e expressão gênica de proteínas que promovem (BAD, BAX) ou protegem (CX36, BCL2, PAX4, e AKT) as ilhotas contra a apoptose.

3-) Determinar os mecanismos da via de sinalização de CNTF em ilhotas pancreáticas através de análise de expressão de proteínas (e de suas formas fosforiladas) envolvidas na vias: JAK/STAT, MAPK e PI3K.

4-) Verificar a expressão do CNTF e de seu receptor, CNTFR, em ilhotas pncreáticas em diversos estágios da vida do animal.

ARTIGO 1

CILIARY NEUROTROPHIC FACTOR (CNTF) PROMOTES SURVIVAL OF NEONATAL RAT ISLETS VIA the BCL-2 ANTIAPOPTOTIC PATHWAY

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Key words: apoptosis, BCL-2, CNTF, neonatal pancreatic islet

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ABSTRACT

Introduction: CNTF belongs to the cytokine family and increases neuron differentiation and/or survival. Pancreatic islets are richly innervated and express receptors for NGFs and may undergo neurotypic responses. CNTF is found in pancreatic islets and exerts paracrine effects in neighbouring cells. The aim of this study was to investigate possible effects of CNTF on neonatal rat pancreatic islet differentiation and/or survival.

Methods: Pancreatic islets from neonatal rats (1-2 d old) were isolated by the collagenase method and cultured for 3 days in RPMI medium with (CNTF) or without (CTL) 1nM of CNTF. Thereafter, glucose-stimulated insulin secretion (RIA), general metabolism by (NAD(P)H production) (MTS), glucose metabolism ($^{14}CO_2$ production), gene (RT-PCR), protein expression (Western blotting), caspase-3 activity (DEVD), and apoptosis (DNA fragmentation) were analysed.

Results: CNTF-treated islets demonstrated reduced glucose-induced insulin secretion. CNTF treatment did not affect glucose metabolism, as well as the expression of mRNAs and proteins that are crucial for the secretory process. Conversely, CNTF significantly increased mRNA and protein levels related to cell survival, such as Cx36, PAX4, and BCL-2, reduced caspase-3 activity, and islet-cells apoptosis.

Conclusion: Our results suggest that CNTF does not affect islet-cell differentiation and, instead, acts as a survival factor reducing apoptosis by increasing the expression of the anti-apoptotic BCL-2 protein, and decreasing caspase-3 activity.

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1. INTRODUCTION

CNTF is a member of the IL-6 family of cytokines that includes IL-11, leukemia inhibitory factor, cardiotrophin-1, oncostatin-M, CNTF and IL-6 itself, all using gp130 as a signal transducing element in the functional receptor complexes and a specific receptor for each of them (CNTF-Rα for CNTF) [1-3]. CNTF is distributed all over the rat central nervous system in neurons and glial cells and also in high concentrations in Schwann cells in the peripheral nervous system. The peptide is known for its neurotrophic effects, being a survival factor for sympathetic, sensory, hippocampal and motor neurons in vitro and in vivo, and in type 2 astrocyte differentiation [2, 4].

Proper control of insulin secretion is crucial for the metabolism of mammals since it exerts a strict regulation of the plasma levels of nutrients, especially glucose. In pancreatic β-cells, a glucose-stimulated increase in the cytosolic ATP/ADP ratio, closes ATP-sensitive potassium (K_{ATP}) channels, which depolarizes the plasma membrane above a threshold, leading to Ca^{2+} entry into the cytosol through activation of voltage-dependent Ca^{2+} channels (VDCCs). The rise in cytosolic Ca^{2+} triggers exocytosis of insulin from secretory vesicles. Type 1 diabetes is characterized by a failure of the immune system that inappropriately recognizes β-cells peptides, leading to an islet infiltration by neutrophiles and a local increase in the concentration of many pro-apoptotic cytokines such as INFγ, TNFα and IL-1β, activating β-cells apoptotic pathways, suppression of protein expression and membrane expression of FAS [5-8]. These effects culminate with an almost complete β-cell loss, leaving other islet-cell types unharmed. The lack of circulating insulin alters the central nervous system's control of nutrient ingestion [9-11] and causes an inappropriate fuel metabolism, with plasma nutrient accumulation and impairment of its intracellular utilization, ultimately leading to organ and system degeneration [12]. These effects may be reversed or, at least, avoided by a proper activation of anti-apoptotic pathways, mainly via BCL-2, the major anti-apoptotic protein in islets [13-16].

CNTF impairs glucose-stimulated insulin secretion (GSIS) and potentiates the inhibitory effect of IL-1β on GSIS, in cultured islets [17]. In addition, it also exhibits many *in-vivo* systemic effects, such as reduction of adiposity, body weight, hyperinsulinemia and hyperglycemia in rats [18-27].

β-Cells express receptors for several neurotrophic factors and may undergo a neurotypic response to neuronal differentiation factors [16, 27, 28]. CNTF is identified in pancreatic β-cells and in the islet-associated nervous system, exerting several actions on non-neuronal cells and may have a paracrine function inside the islets. Furthermore, CNTF expression has already been identified in β-cells [17]. For these reasons, we decided to study the possible effects of CNTF on rat pancreatic islets differentiation and/or survival.

2. MATERIAL AND METHODS

2.1. Chemicals

D-[U-^{14}C]glucose and ^{125}I-insulin were from G.E. Health Care; aminotransferase inhibitor amino-oxyacetate (carboxymethoxylamine) and Sybr-Green were from Sigma-Aldrich; MTS/PMS preparation was from CellTitter96 aqueous assay (Promega), and all RT-PCR reagents were from Invitrogen. Other reagents were from Sigma, whenever specified.

2.2. Islets isolation and culture

Neonatal (1-2 d old) Wistar rats were from the State University of Campinas animal facilities. After decapitation, the islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreas in Hanks balanced salt solution (137 mM NaCl, 5.5 mM KCl, 4.5 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, pH 7.4). Islets were extensively washed in sterile Hanks solution and cultured in RPMI 1640 medium supplemented with 2g/l $NaHCO_3$, 1% (v/v)

penicillin/streptomycin, 5.6 mM D-glucose and 2% Fetal Bovine Serum, pH 7.4. Approximately 1000 islets/dish were maintained at 37°C in a humidified atmosphere with 3% CO_2 for 3 days in the presence or absence of 1 nM CNTF. The medium was renewed every 24 h. Islet experimental groups were assigned according to culture conditions: CTL (Control Group, islets cultured without CNTF) and CNTF (Treated Group, islets cultured in presence of CNTF).

2.3. Insulin secretion

Batches of 10 islets each were incubated in Krebs-Hepes buffered saline (KHBS, in mM: 115 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 15 Hepes) containing 0.5 g/l BSA and 5.6 mM glucose, pH 7.4, and equilibrated with 95% O_2 and 5% CO_2 for 30 min in 37^{o}C. The medium was discarded and the islets incubated for a further period of 1 h in 1 ml of KHBS containing 2.8 or 16.7 mM glucose. The supernatant was collected and insulin was measured by radioimmunoassay.

2.4. Glucose metabolism

Batches of 50 islets each were incubated for 2 h at 37^{o}C in KHBS containing 2.8 or 16.7 mM glucose with trace amounts of either D-[U-^{14}C]glucose for $^{14}CO_2$ production. The batches were added with HCl 1N to stop respiration and the $^{14}CO_2$ collected for 1h in 4^{o}C 1M NaOH.

2.5. RT-PCR

Groups of 1000 islets were homogenized in Trizol following phenol-chloroform RNA extraction, according to the manufacturer's instructions. RNA integrity was asserted through agarose gel. Reverse transcriptase reaction was performed using 3 µg of total RNA. The reactions were incubated for 5 min at 65^{o}C before addition of 150 ng random primers, for 10 min at 25^{o}C before addition of 14.3 mM $MgCl_2$, 2.8 mM DTT, 0.4 U/µl RNase-out and at 42^{o}C for 2 min before addition of

1.25 U/µl RNA Super Script II. Samples were incubated at 42°C for 50 min, at 70°C for 15 min, and then cooled to 4°C. The cDNAs obtained were diluted in PCR buffer (60 mM Tris-HCl, 1.5 mM $MgCl_2$, 15 mM NH_4SO_4, pH10) with 50 mM $MgCl_2$, 0.3 mM each of dATP, dCTP, dGTP and dTTP, 2.5 U/µl Taq DNA polymerase (Gibco/BRL), 10 mM forward primer and reverse primer were then added. PCR amplification of cDNA was performed with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR program employed the following cycle profile: 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min, extension for 1.5 min at 72°C, and maximization of strand completion for 7 min at 72°C. The annealing temperatures and the number of cycles used in each amplification are shown in the legends. Following amplification, the cDNA fragments were analyzed on 1.6 % agarose gels containing a 100 bp DNA molecular weight ladder (Gibco/BRL). PCR products were analyzed by ethidium bromide UV fluorescence in a Gel Doc EQ analyzer (Bio-Rad).

Internal controls for reactions were chosen among various controls used; of these, the RPS-29 subunit of the 40S small ribosomal unit showed the best homogeneity between treated and non-treated groups. Primers were designed and tested against *Rattus norvergicus* genome (Gene Bank) to ensure no amplification of other cDNAs. The Sense (S) and Anti-sense (AS) oligonucleotide primers used were as follows: RPS-29 (S) 5'AGG CAA GAT GGG TCA CYCLE CCA GC3'; RPS-29 (AS) 5'AGT CGA ATC CAT TCA CYCLE GGT CG3'; Rat pro-insulin 2 (S) 5' TTG CAG TAG TTC TCC AGT T 3'; Rat pro-insulin 2 (AS) 5' ATT GTT CCA ACA TGG CCC TGT 3'; GLUT2 (A) 5'CAT TGC TGG AAG CGT ATC AG3'; GLUT2 (AS) 5'GAG ACC TTC TGC TCA CYCLE GTC GAC G3'; PKCα (S) 5' CCT GCT CTA CGG ACT TAC T, PKCα (AS) 5' TGT AGT ATT CAC CCT CCT C; NKX 6.1 (S) 5' AAA CAC ACC AGA CCC ACA TTC TC NKX6.1 (AS) 5' TTC TCG TCG TCA GAG TTC GG; Glucokinase IV (S) 5' ATG AAG ACC GCC AAT GTG AGG Glucokinase IV TGT TGT GGA TCT GCT TTC GGT C; CX36 (S) 5' AGT GGT GGG AGC AAG CGA GAA G; CX36 (AS) 5' ACA ACC CTG GGA CAC TGA AGC C; PAX4 (S) 5' ACC AGC CAC AGG AAT CGG AC

PAX4 (AS) 5’ AAG CCA CAG GAA GGA GGG AG; BAD (S) 5’ CAG TGA TCT GCT CCA CAT TC BAD (AS) 5’ ATG ATA GGA CAG CAC CCA GT; BAX (S) 5’ AAG AAG CTG AGC GAG TGT CT, BAX (AS) 5’ CAA AGA TGG TCA CTG TCT GC; AKT (S) CCT CAA GTA CTC ATT CCA GAC AKT (AS) CTC ATA CAC ATC TTG CCA CAC; BCL2 (S) 5’ GTA TGA TAA CCG GGA GAT CG, BCL2 (AS) 5’ AGC CAG GAG AAA TCA AAC AG. All annealing temperatures and number of cycles were chosen to agree maximal sensibility to sample cDNA content.

2.6. Western-Blotting

After culture, groups of islets were pelleted by centrifugation and then resuspended in 50-100 µl of homogenization buffer containing protease inhibitors, as described. The islets were sonicated (15 s) and the protein was determined by the Bradford method (36) using bovine serum albumin as standard. The samples volume was adjusted to provide the same amount of protein added to each lane. Samples containing 70 µg of protein from each experimental group were separated by SDS-PAGE, transferred to nitrocellulose membranes and stained with Ponceau S. No differences in the total amount of protein were observed as judged by densitometric analysis of the stained membranes (not shown). CX36 was detected in the membrane after 2 h incubation at room temperature with a rabbit polyclonal antibody against CX36 (Zymed, diluted 1:1500 in TBS plus 30 g/L dry skimmed milk), and BCL-2 with a rabbit polyclonal antibody against BCL-2 (Santa Cruz, diluted 1:500 in TBS plus 30 g/L dry skimmed milk). Detection was performed using enhanced chemiluminescence (SuperSignal West Pico, Pierce) after incubation with a horseradish peroxidase-conjugated secondary antibody. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD) of the developed autoradiogram.

2.7. NAD(P)H determination

NAD(P)H was measured by the coupled reduction of phenazine methosulfate (PMS) and subsequent transfer of electrons to the tetrazolium salt, MTS, both of which are membrane permeable.

Mixing of MTS and PMS solutions was carried out according to the manufacturer's instructions. Little change in 492 nm absorbance was seen with concentrations of NAD(P)H below 10 μM. Due to the significant interference of proteins bound to NAD(P)H, standard curves were less accurate at low concentrations of NAD(P)H. However, spectroscopic analysis demonstrated an increase of ~10^{-3} cm^{-1} $islet^{-1}$ light absorption at 650 nm when different numbers of islets were disrupted in a solution of MTS/PMS containing 2 mg/ml BSA. Heat-denatured islet homogenates were used as negative controls.

Static measurements of NAD(P)H were performed by incubating groups of 200 islets in KHBS containing 2.8 or 16.7 mM of glucose, reproducing the same conditions used in insulin secretion experiments. Islets were then washed in ice-cold Hanks solution and immediately disrupted by sonication in 150 μl Hanks solution. Homogenates were centrifuged at 10,000g for 2 min to remove islet debris. Supernatants were then added to the MTS/PMS solution and incubated for 30 min at room temperature before recording absorbance at 650 and 405 nm (background). Samples with no islet were used as blanks.

Dynamic measurements of NAD(P)H were carried out by incubating groups of 20 islets in 200 μl of KHBS containing 0 or 20 mM of glucose and 5% (v/v) of MTS/PMS. This concentration of MTS/PMS was chosen after testing concentrations from 1 to 20% (v/v) and searching for the best sensitivity. As judged by a transient rate of MTS/PMS reduction, concentrations of PMS/MTS above 7% caused cell death due to NAD(P)H depletion. Samples were incubated for 3 h under 95% O_2 + 5% CO_2 atmosphere (140 μM of O_2 in KHBS). Samples with no islets were used as blanks and 492 nm absorbance values were recorded every 30 min. NADPH standard curves were used to calculate the reduced amounts of NAD(P)H in samples. The NAD(P)H reduction rate (NRR) values were taken from the temporal increase of MTS/PMS absorbance in each sample.

2.8. Caspase-3 activity assay.

The colorimetric method of cleavage of the DEVD was employed. Control and treated incubated neonatal rat islets were lysated in Chaps-containing hypotonic buffer, centrifuged at 12,000 rpm, and the supernatants were stored at -80 °C for further measurement. For the assay, up to 50 µl of the lysate (corresponding to 100 µg of protein), 50µl of substrate, and enough volume of assay buffer for completing 100 µl of total sample assay volume, were placed in clear flat bottom 96 well plates. The absorbance was measure at 405 nm, reading every 5 min for 2 h at 37 °C. Total protein content was determined by the Bradford assay. The specific activity of caspase-3 was calculated according to the manual, using a known standard pNA solution.

2.9. DNA fragmentation assay

DNA was isolated from neonatal rat islets, separated in fragmented and integral subunits by trizol/triton method. Both were quantified by Sybr-green method, as ng/ml of DNA. Data are expressed as Fragmented/Total DNA.

2.9. Statistics

Point-to-point comparisons were made by Students t-test. Groups were compared by two-way ANOVA using unpaired the Tukey-Kramer method as post-test. Results were considered significantly different if $P<0.05$. In RT-PCR experiments, results were considered different only if $P<0.001$.

3. RESULTS

The Glucose-Stimulated Insulin Secretion (GSIS) from islets cultured in the presence of 1 nM CNTF for 3 days, subsequently incubated for 1 h in Krebs-bicarbonate solution at 2.8 or 16.7 mM

glucose, was significantly lower compared with control islets (P<0.05) (Fig. 1). The total insulin **(CTL 2.8** - 291,38 ± 60,99; **CNTF 2.8** - 293,20 ± 31,42; **CTL 16.7** - 334,62 ± 43,41; **CNTF 16.7** - 336,78 ± 23,32 ng/islet, n = 6) and DNA (**CTL** - 1.82 ± 0.06 **CNTF** - 1.86 ± 0.07 ng/ml, n = 4) islet contents were similar between groups**.** In addition, a small reduction of the mitochondrial metabolism was observed in the CNTF-treated islets, as judged by the NADPH reduction rate (P<0.05 vs CTL) (Fig. 2), suggesting that CNTF-treatment does not improve the maturation of the islet secretory process. To assess whether these inhibitory effects were due to an altered glucose metabolism, we measured the glucose utilization by CNTF and CTL islets. No differences in $^{14}CO_2$ production, at basal and stimulatory concentrations of glucose, were observed between groups (Fig. 3).

To further analyze the effects of CNTF on the maturation of neonatal islets, we investigated the transcription level of several proteins involved in this process, such as: GLUT2 [29-31], Insulin, PKCα [32-35], PDX-1 [36-38], NKX6.1 [39], and Glucokinase IV [40]. None of the mRNA analyzed was significantly altered by CNTF-treatment (Fig. 4 A-F). The protein expression for some of these genes confirmed the results obtained by RT-PCR (Fig. 5 A-B). Indicating that CNTF has no effect on islet-cells differentiation.

In the next series of experiments, the expression of genes encoding proteins related to cells survival were analyzed, such as the pro-apoptotic BAD [13] and BAX [14] and the anti-apoptotic Akt [41], Bcl-2 [41-47], Cx36 [48-51], and PAX4 [52-53] genes. No differences were observed between CNTF and CTL islets for BAD, BAX, and Akt (Fig. 6A-C) genes, whereas the Bcl-2, Cx36, and PAX4 genes (Fig. 6D-F) were significantly higher in the CNTF-treated islets. The expression of two transcripts (Bcl2 and Cx36) was confirmed by Western blotting for the corresponding proteins (Fig. 7 A-B), confirming that the peptide acts in neonatal rat islets as a survival factor.

Finally, the effect of CNTF on the final steps of cell death was assessed by evaluating the caspase-3 activity, an accurate marker for apoptosis [54-57], and islet-cells DNA fragmentation [46]. CNTF-treatment significantly reduced the islet caspase-3 activity (Fig 8A) and DNA fragmentation (Fig. 8B) compared with CTL islets. These results clearly indicate a lower level of apoptosis and, therefore, increased islet survival promoted by CNTF.

4. DISCUSSION

It has been suggested that CNTF, released from destroyed β-cells during the inflammatory process that occurs during the onset of type 1 diabetes, may act as a pro-inflammatory cytokine by potentiating the action of IL-1β on β-cells [17]. However, to date, CNTF, in contrast to IL-6 [27] has not yet been tested as a differentiation and/or survival factor in pancreatic islets. The present results show that CNTF impairs GSIS in cultured islets, but promotes their survival by reducing apoptosis.

Basal and glucose-stimulated insulin secretions have been suggested to be accurate markers of differentiated and/or functionally mature pancreatic islets. Here, we show that mitochondrial islet metabolism was significantly reduced in CNTF-treated islets, whilst glucose metabolism remained unaltered; indicating that islets demonstrate a lower activity and responsiveness, but that they are integral, features typical of undifferentiated cells. Furthermore, the expressions of major proteins related to differentiated β-cells, such as insulin, Glut2, PKC, PDX-1, NKX6.1, and Glucokinase IV were unaffected by CNTF treatment. The possible effects of CNTF on islets survival had yet to be investigated, thus, we first evaluated the mRNA levels of a number of apoptosis-related proteins, including BAD, BAX, AKT, Bcl-2, Cx36, and PAX4. No changes in BAD, BAX and AKT expression were observed; in contrast, Bcl-2, Cx36, and PAX4 mRNA levels were significantly higher in CNTF-treated islets, an effect subsequently confirmed by Western-blotting of two of the referred proteins. Due

to the anti-apoptotic function, attributed to these proteins, particularly Bcl-2, these results suggest a probable survival effect of CNTF in pancreatic islets.

The final steps of apoptosis involve the activation of caspase-3 in the cytosol and, depending on the degree of activation, the process is virtually irreversible. Thus, the reduced caspase-3 activity associated with the lower islet-cells DNA fragmentation, observed in CNTF-treated islets, are markers of increased islets survival promoted by the peptide. The varying results observed following CNTF-treatment in islets might be due to the increase in Bcl-2 expression; it has been suggested that in addition to its anti-apoptotic effects, Bcl-2 may have a role in regulating metabolism, and many findings support this theory. Bcl-2 is involved in regulation or generation of ROS [58, 59], can alter mitochondrial matrix volume or structure [60], permeability to or consumption of ATP [61], permeability of VDAC [62], and sensitivity of the MPT to Ca^{2+} [63].

Given the strict relationship between these parameters and metabolic function, it may be proposed that Bcl-2 affects NADH reduction, an accurate indicative of metabolism, and this is exactly what we observed in the present study; a significantly lower NADH reduction rate that suggests a decrease in general metabolism, without affecting glucose metabolism.

We hypothesize that the observed CNTF effects could be explained by the increased Bcl-2 expression, leading on one hand, to a lower generation of ROS and the inhibition of apoptotic pathways, a subsequent reduced caspase-3 activity and a lower apoptosis rate. On the other hand, Bcl-2 reduced the mitochondrial metabolism (as evaluated by NADH reduction rate).

Alternatively to previous proposals [17], we suggest that the effect of CNTF in IL-1β action on pancreatic islets may be due to a parallel rather than a synergic pathway. Our findings support the

idea that CNTF acts as an anti-apoptotic cytokine that protects islets against the inflammatory processes by increasing Bcl-2 expression and promoting its survival.

In conclusion, CNTF impairs GSIS, as well as the mitochondrial metabolism of pancreatic islets, and has no effect on glucose metabolism and the expression of genes and proteins related to pancreatic islet insulin secretion. Instead, the present data indicate that CNTF acts as a repressor of differentiation (an effect already described for the peptide), being an effective promoter of islet survival by enhancing the levels of survival proteins, especially Bcl-2. We hypothesize that during inflammatory processes, CNTF, present in the islets or in the associated peripheral nervous system, acts as a survival factor for the neighboring islets during the early stages of lesion. CNTF has not been tested in an animal model for IDDM, mainly due to its harsh side effects, such as cachexia and anorexia, observed in other animal models. Thus, new methods of delivering CNTF to target cells to avoid its side effects may potentiate CNTF as an important therapeutic tool.

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Figure 1

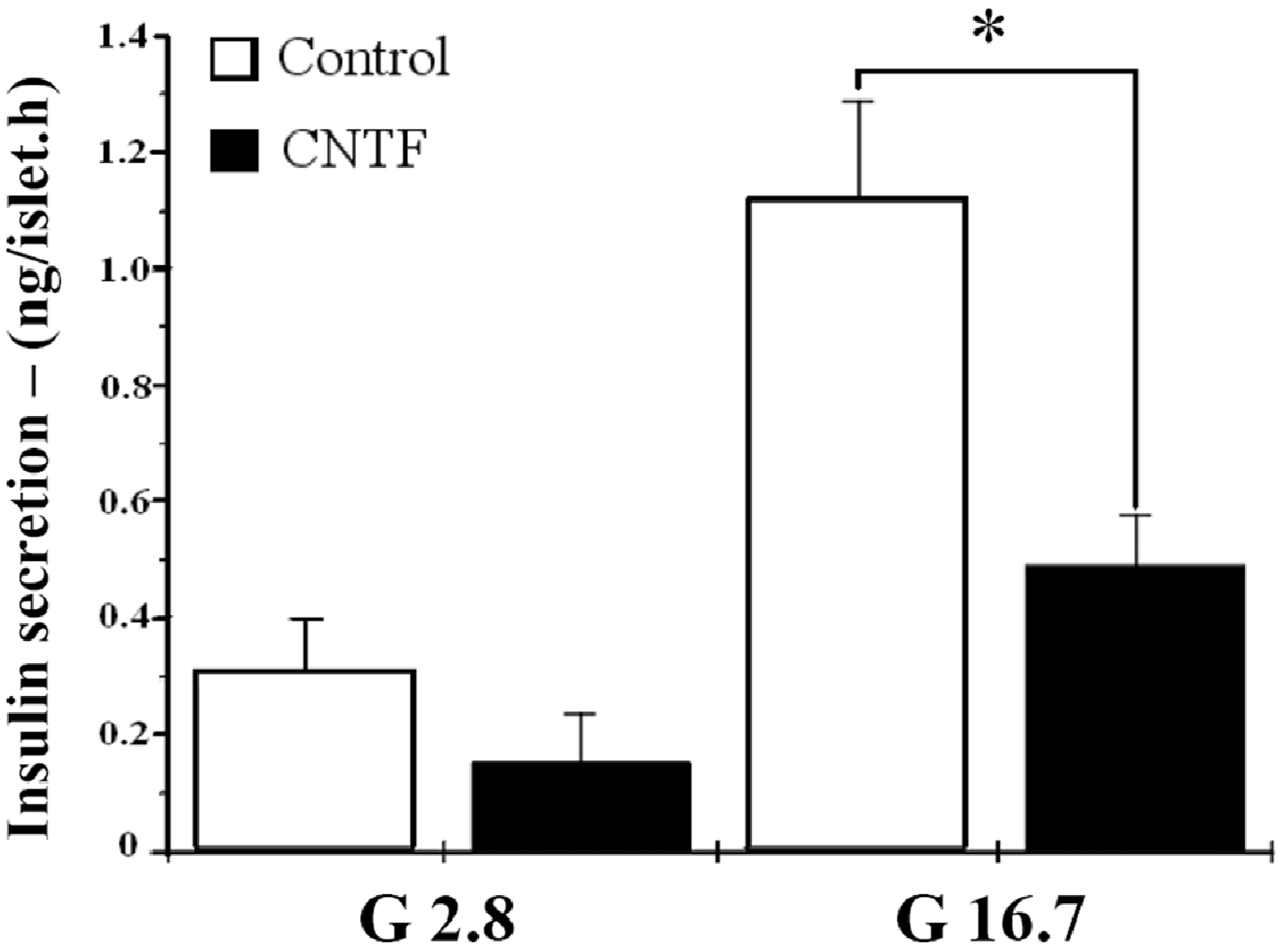

Fig. 1. Insulin secretion in islets cultured for 3d with 5.6 mM, 2% FBS and 1% Penicillin, in the presence or absence of 1nM CNTF (CNTF and Control groups, respectively). Islets were pre-incubated for 30 min in KHBS with 5.6 mM glucose, as described, and then incubated either with 2.8 or 16.7 mM glucose for 1 h. Bars are means ± SEM of 8 independent experiments. * $P < 0.05$.

Figure 2

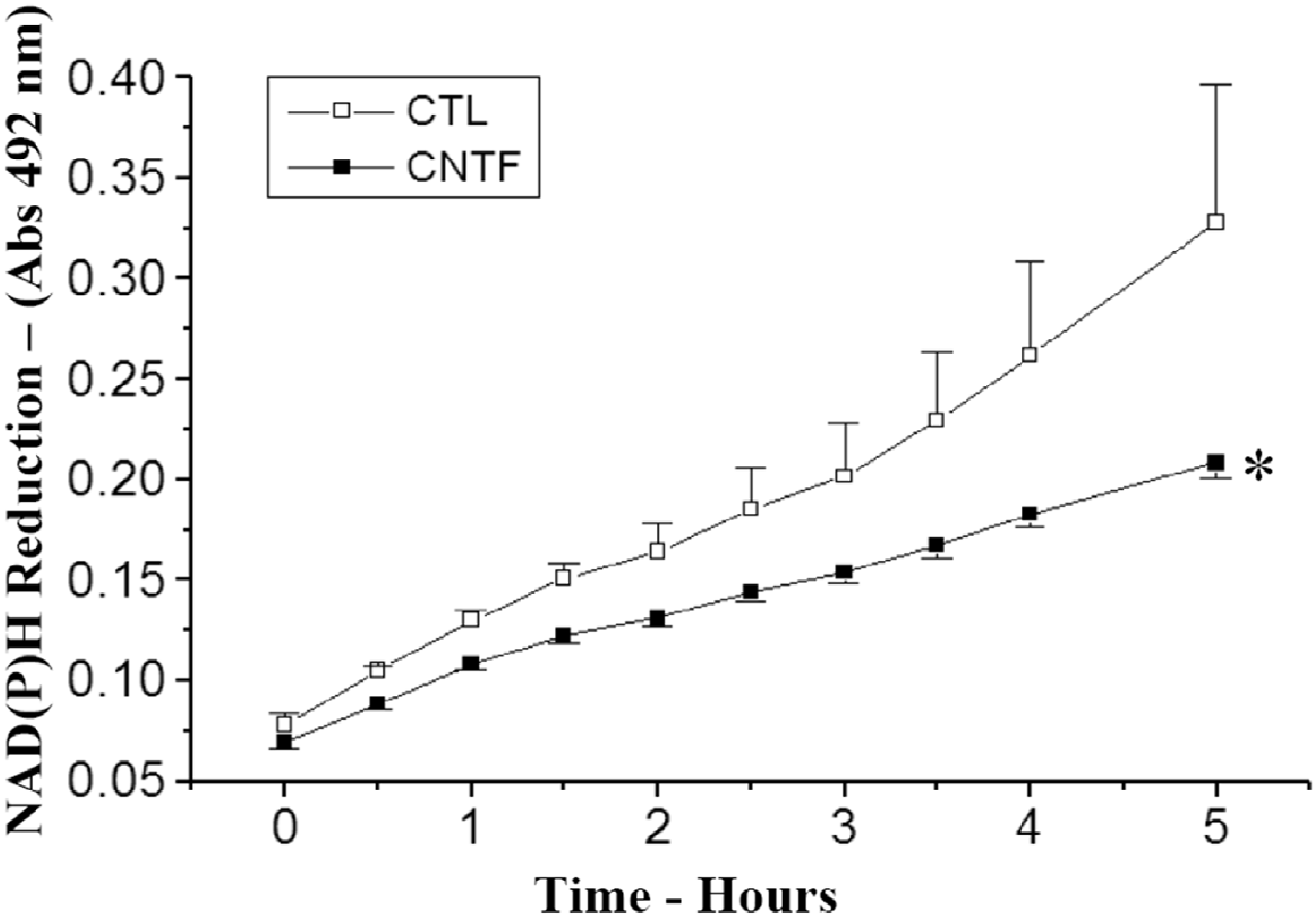

Fig. 2. Islet metabolism, as evaluated by NAD(P)H reduction rate (NRR). Islets were incubated for 3h in KHBS containing 5% (v/v) of MTS/PMS and 10 mM glucose. Incubations were performed in a 95% O_2 atmosphere that produced 140 μM O_2 in KHBS solution. NRR of each sample was calculated as the temporal change in NAD(P)H, causing MTS/PMS reduction. Values are means ± SEM of 10 experiments. * $P<0.05$.

Figure 3

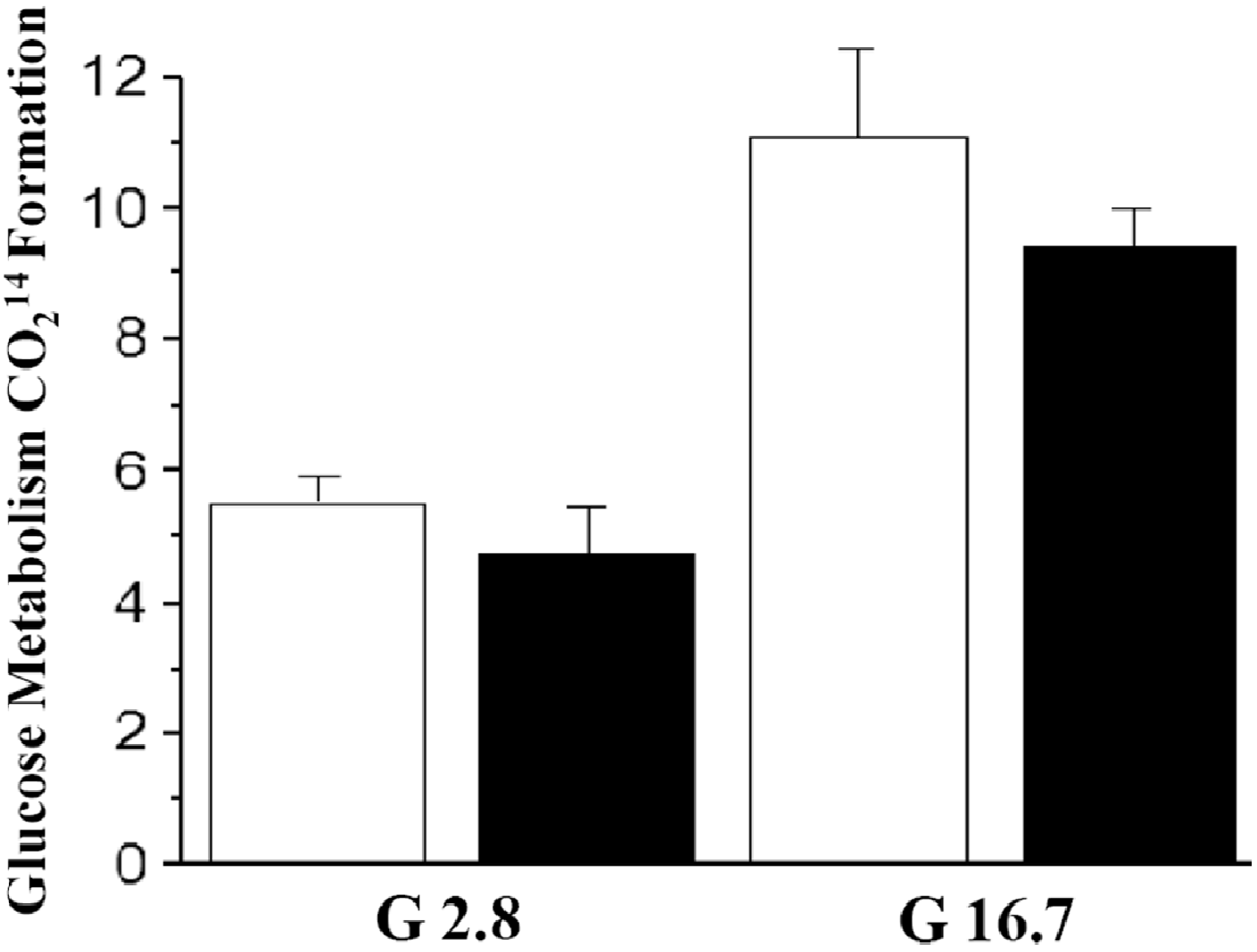

Fig. 3.Glucose oxidation of islets cultured for 3d with 5.6 mM glucose, 2% FBS and 1% Penicillin, in the presence or absence of 20ng/ml CNTF (CNTF and CTL groups, respectively). Islets were incubated for 120 min in KHBS with 2.8 or 16.7 mM glucose containing equal amounts of D[U-^{14}C]glucose to measure $^{14}CO_2$ production. Bars are means ± SEM of at least 8 experiments. * $P<0.05$ related to each respective control (2.8 mM glucose).

Figure 4

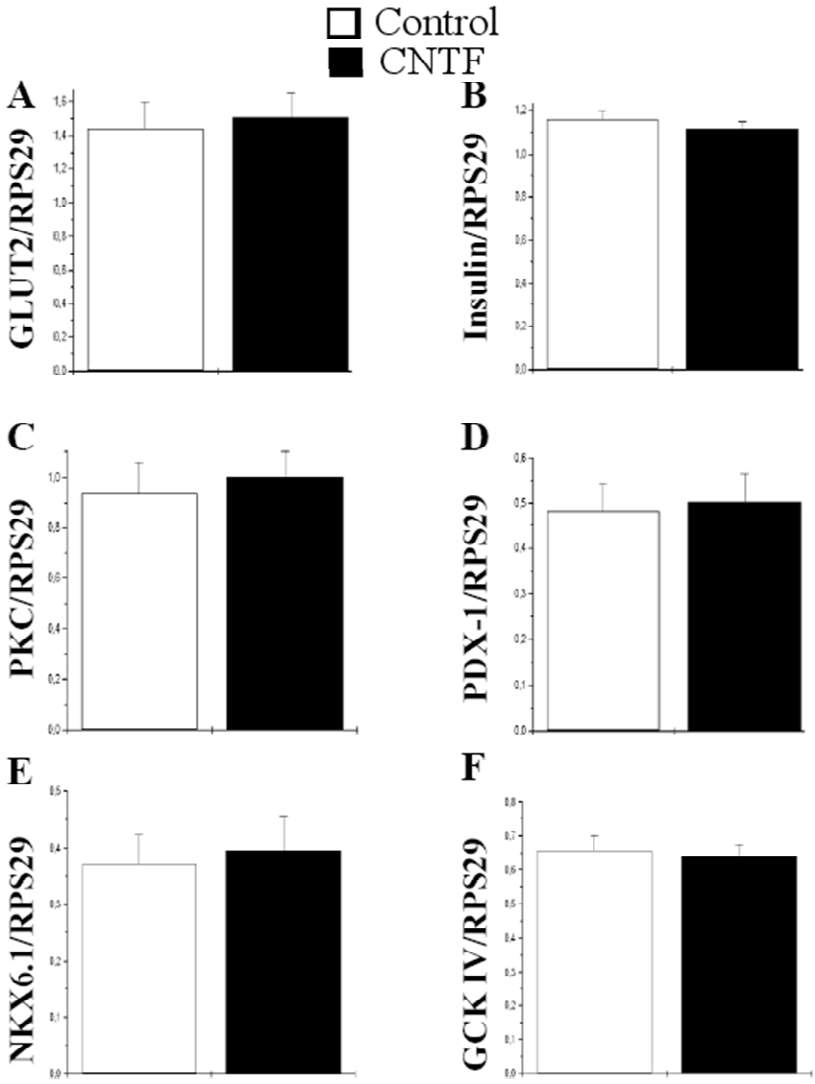

Fig. 4. Effect of culture with 1nM CNTF for 3d on mRNA levels, as evaluated by RT-PCR, of GLUT-2 (**A**), Insulin (**B**), PKC (**C**), PDX-1 (**D**) NKX6.1 (**E**) and Glucokinase IV (GCK IV) (**F**). RT-PCRs annealing temperatures and cycle numbers used were as follows: 55º C and 29 cycles for GLUT-2; 57º C and 23 cycles for Insulin; 57º C and 31 cycles for PKC; 55º C 29 cycles for PDX-1; 60º C and 30 cycles for NKX6.1; 60º C and 30 cycles for GCK IV. RPS-29 was used as an internal control (57º C and 29 Cycles), showing no variation amongst the conditions tested. Plotted columns are means ± SEM of 12 experiments. * $P<0.001$

Figure 5

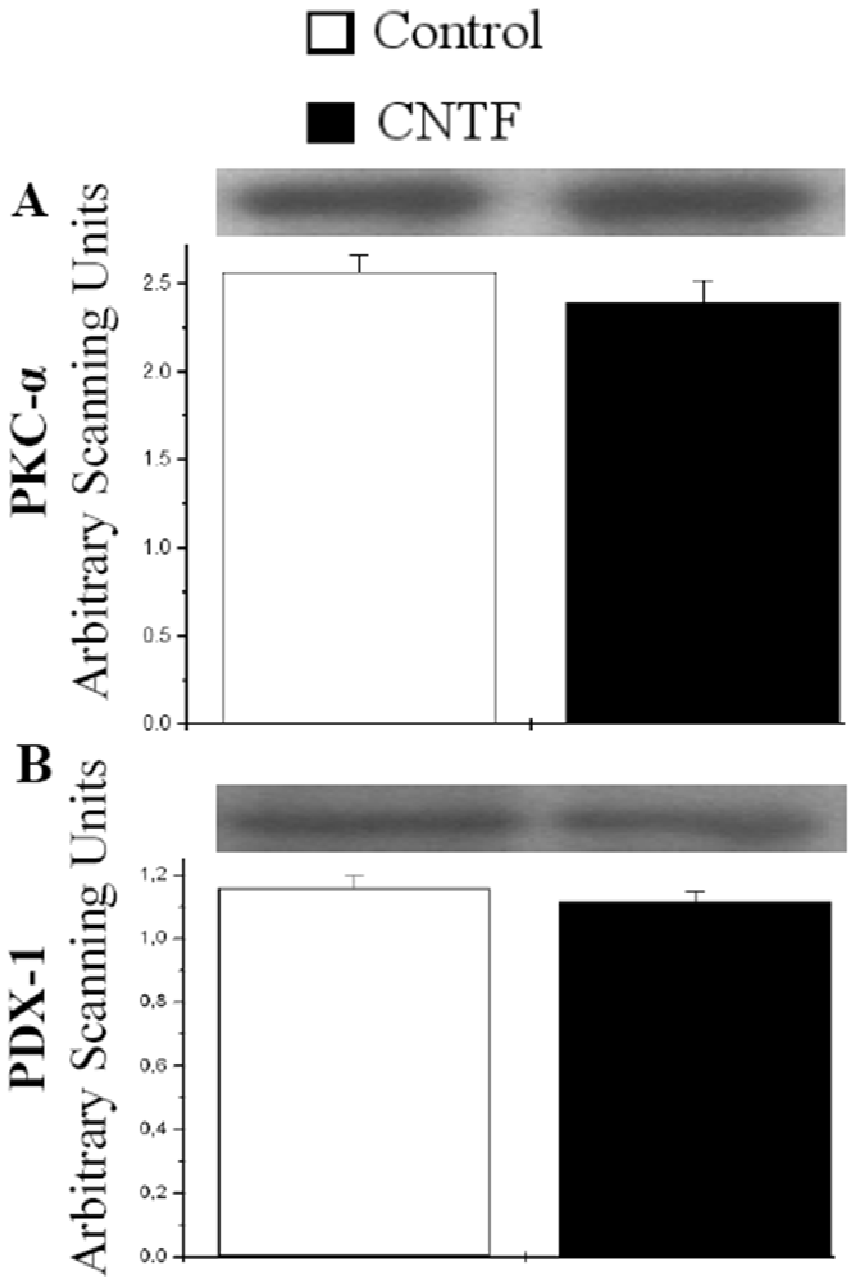

Fig. 5. Protein expression, as measured by Western blotting of PKC (**A**), PDX-1 (**B**) in neonate rat islets cultured for 3 d in the presence (CNTF■) or absence (Control □) of 1nM CNTF. The values are the means ± SEM of six independent experiments. * P<0.05

Figure 6

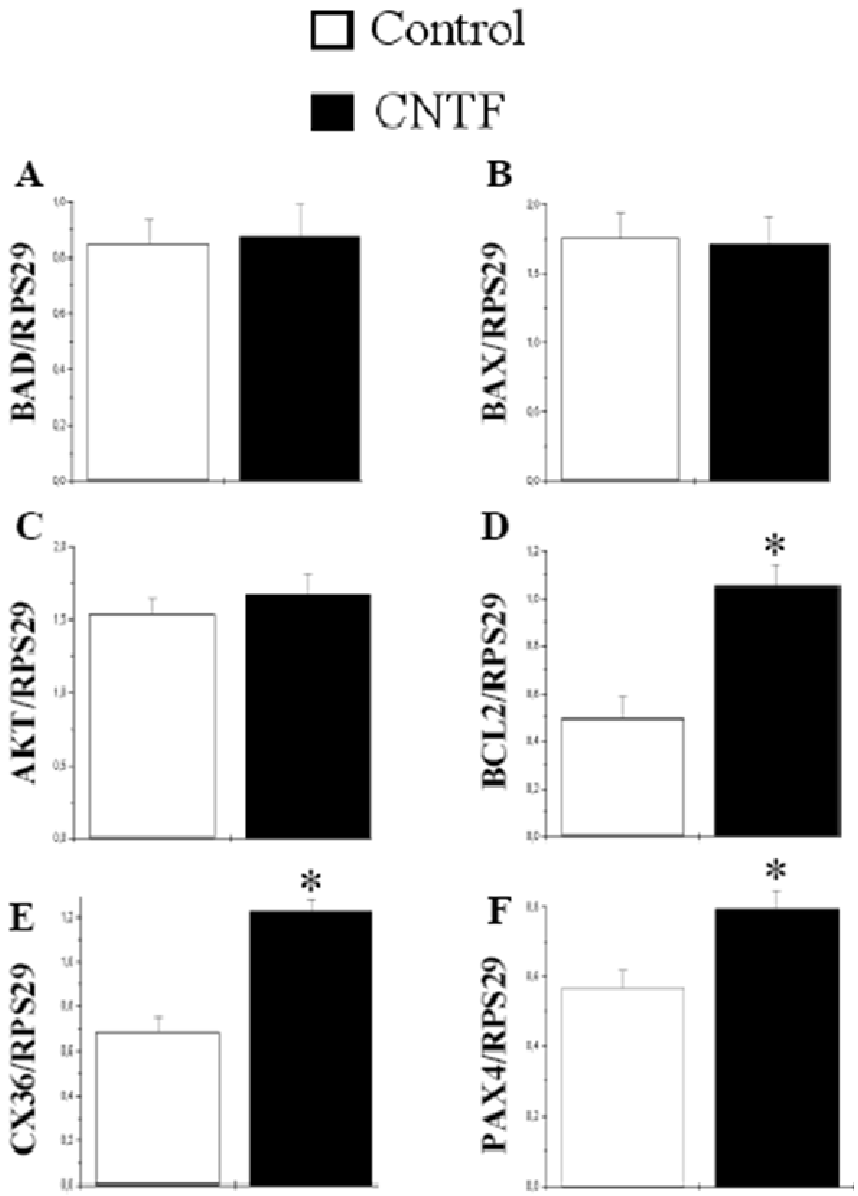

Fig. 6. Effect of 3d culture with 20ng/ml CNTF on mRNA levels, as evaluated by RT-PCR, of BAD (**A**), BAX (**B**), AKT (**C**), BCL-2 (**D**), Cx36 (**E**) and PAX4 (**F**). RT-PCRs annealing temperatures and cycle numbers used were as follows: 60ºC and 29 cycles for BAD; 59º C and 31 cycles for BAX; 59º C and 30 cycles for AKT and 61ºC; 32 cycles for BCL-2; 57º C and 31 cycles for CX36; 62º C and 31 Cycles for PAX4. RPS-29 was used as an internal control (57ºC and 29 cycles), showing no variation amongst the conditions tested. Plotted columns are means ± SEM. of 12 experiments. * P<0.001.

Figure 7

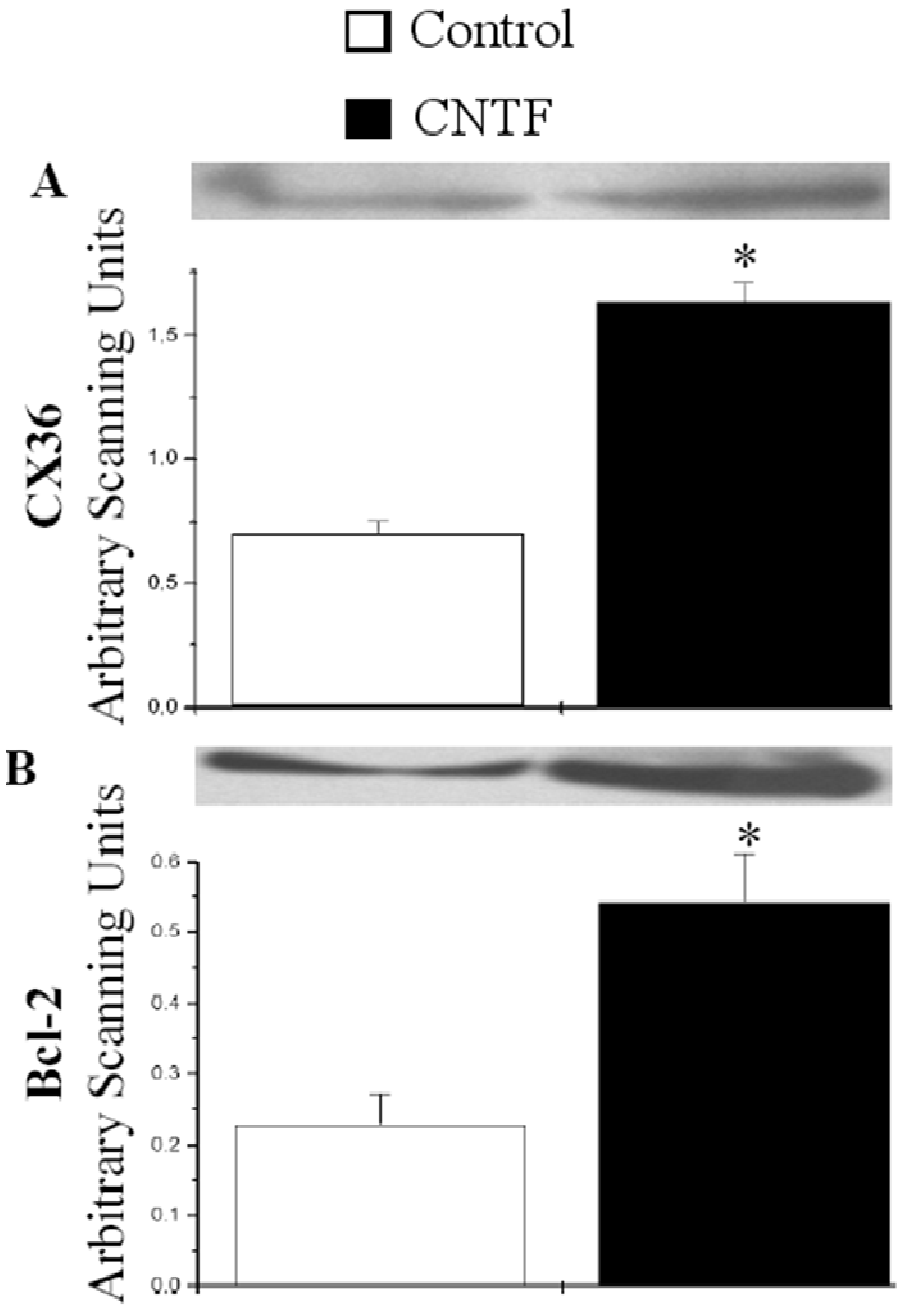

Fig. 7. Protein expression, as measured by Western blotting of Cx36 (**A**) and Bcl-2 (**B**) in neonate rat islets cultured for 3d in presence (CNTF ■) or absence (Control □) of 1nM CNTF. The values are the means ± SEM of six independent experiments. * $P<0.05$.

Figure 8

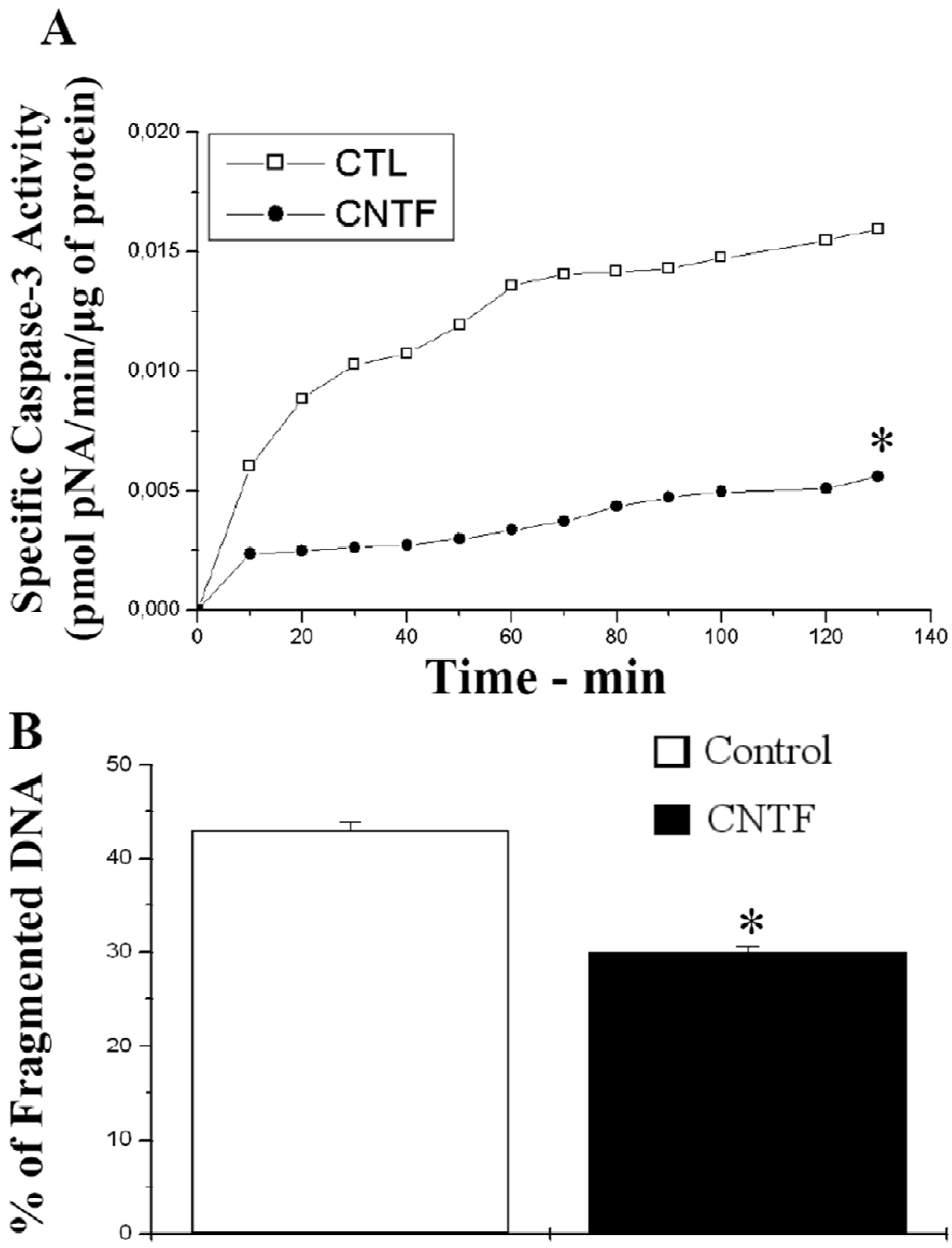

Fig. 8. Caspase-3 Activity (**A**) and Percentage of DNA fragmentation (**B**) in neonate rat islets cultured for 3d in presence (CNTF ■) or absence (Control □) of 1nM CNTF. The values are the means ± SEM of six (**A**) or four (**B**) independent experiments. * $P<0.05$.

ARTIGO 2

CILIARY NEUROTROPHIC FACTOR (CNTF) SIGNALS THROUGH STAT3-SOCS3 PATHWAY AND PROTECTS RAT PANCREATIC ISLETS FROM CYTOKINE-INDUCED APOPTOSIS

CNTF signals through STAT3-SOCS3 in rat islets

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Key words: Type 1 diabetes, STAT3, CNTF, pancreatic islets, SOCS3, Apoptosis.

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ABSTRACT

CNTF is a cytokine that promotes survival and/or differentiation in many cell types, including rat pancreatic islets. In this work, we studied the mechanism of CNTF signal in neonatal rats pancreatic islets isolated by the collagenase method and cultured for 3 days in RPMI medium without (CTL) or with 1nM of CNTF (CNTF). The medium contained, when necessary, specific inhibitors of the PI3K, MAPK and JAK/STAT3 pathways. mRNA expression (RT-PCR) and protein phosphorylation (Western blot) of Akt, ERK1/2 and STAT3, and SOCS-3 (RT-PCR and Western-blot), as well as glucose-stimulated insulin secretion (GSIS) (Radioimmunoassay), were analyzed. Our results showed that Akt, ERK1 and STAT3 mRNA expression, as well as phosphorylated Akt and ERK1/2, was not affected by CNTF treatment. CNTF increased cytoplasmatic and nuclear phosphorylated STAT3, and the SOCS3 mRNA and protein expression. In addition, CNTF lowered apoptosis and impaired GSIS. These effects were blocked by the JAK inhibitor, AG490 and by the STAT3 inhibitor Curcumin, but not by the MAPK inhibitor, PD98059, nor by the PI3K inhibitor, Wortmannin. In conclusion, CNTF signals through the JAK2/STAT3 cascade, increases SOCS3 expression, impairs GSIS and protects neonatal pancreatic rat islets from cytokine-induced apoptosis. These findings indicate that CNTF may be a potential therapeutic tool against Type 1 and/or Type 2 Diabetes.

INTRODUCTION

Proper control of insulin secretion by β-cells is crucial for the metabolism of mammals, since it exerts a strict regulation of the plasma levels of nutrients, especially glucose. A glucose-stimulated increase in the cytosolic ATP/ADP ratio closes the ATP-sensitive potassium (k_{ATP}) channels, which depolarise the plasma membrane above a threshold, leading to Ca^{2+} entry into the

cytosol through voltage-dependent Ca^{2+} channels; the rise in cytosolic Ca^{2+} triggers exocytosis of insulin from secretory vesicles. Type 1 and type 2 diabetes are the consequence of destruction of pancreatic β-cells or the impairment of insulin secretion, respectively. The resulting lack of circulating insulin alters the central nervous system's control of nutrient ingestion [1, 2, 3], causing an inappropriate fuel metabolism, with plasma nutrient accumulation and impairment of its intracellular utilization, ultimately leading to organ and system degeneration [4].

CNTF is a member of the IL-6 family of cytokines, which includes IL-11, leukaemia inhibitory factor, cardiotrophin-1, oncostatin-M, CNTF and IL-6 itself, all using gp130 as a signal transducing element in the functional receptor complexes and a specific receptor for each of them (CNTF-Rα for CNTF) [5, 6, 7]. CNTF is distributed all over the rat central nervous system, in neurons and glial cells, and also in Schwann cells in the peripheral nervous system [8].

The peptide is known for its neurotrophic effects, being a survival factor for sympathetic, sensory, hippocampal and motor neurons, *in vitro* and *in vivo* [8, 9, 10, 11, 12, 13, 14, 15] and also as a differentiation factor in type 2 astrocytes [7, 16]. Although CNTF impairs glucose-stimulated insulin secretion (GSIS)[17, 18], the peptide is suggested to be anti-diabetogenic, exhibiting many *in-vivo* systemic effects, such as reduction of adiposity, body weight, hyperinsulinemia and hyperglycemia in rats, amongst other actions. [19, 20, 21, 22, 23, 24, 25]. In addition, CNTF promotes the survival of rat pancreatic islets, albeit not promoting its differentiation [18].

It has been established that, in most cell-types, CNTF signals through Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3). CNTF binding to CNTFR on the gp130 complex activates the receptor-associated kinase JAK2 [26] and phosphorylates tyrosine residues on CNTFR, recruiting and phosphorylating STAT3, which dimerizes and translocates to the nucleus to regulate gene transcription [27, 28]. However, CNTF also signals through phosphatidylinositol-3-OH

kinase (PI3K) [24, 29] or mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/ERK) [30] pathways, either concomitantly or independently of JAK/STAT3 signaling.

Activation of JAK/STATs, as a whole, stimulates cell proliferation, differentiation, migration and inhibits apoptosis; STAT3 is not an exception, and has been described as an anti-inflammatory, anti-apoptotic and pro-survival pathway [31, 32]. Regulation of the STAT3 pathway involves multiple mechanisms, such as: STAMs (Signal-transducing adapter molecules) [33], StIP (Stat Interacting proteins) [34], Lnk/APS family [35], PIAS (protein inhibitors of activated stats) and PTPs (protein tyrosine phosphatases), and SOCS (suppressors of cytokine signaling)[36].

Among these, SOCS3 has a particular relevance in pancreatic islet β-cells, since it is involved in many processes, including the protection of islets from IL-1β-induced toxicity [37, 38], resistance to STZ-induced type 1 diabetes [39], regulation of β-cell mass and proliferation [40], differential gene expression [41] and impairment of insulin secretion [42]. SOCS3 inhibits CNTF signaling [43, 44] by the interaction of its SH2 domain with the cytoplasmic domain of the phosphorylated gp130 common receptor subunit [44,45].

In this study, we aimed to clarify the CNTF signaling pathways in rat pancreatic islets and their possible protective effects on cytokine-induced pancreatic islet apoptosis. Our results indicate that CNTF impairs GSIS and protects neonatal pancreatic rat islets from cytokine-induced apoptosis, activating the JAK/STAT3 cascade and increasing SOCS3 expression.

MATERIAL AND METHODS

Reagents

^{125}I-insulin was bought from G.E. Health Care, all RT-PCR reagents were from Invitrogen, Wortmannin was from Sigma-Aldrich, PD98059 was from InvivoGen, Curcumin was from

Cayman and AG490 from Calbiochem, La Jolla, CA, USA. Other reagents were from Sigma, whenever specified.

Islet Isolation and Culture

Neonatal (1-2 d old) Wistar rats were from the State University of Campinas animal facilities. After decapitation, the islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreas in Hanks balanced salt solution (137 mM NaCl, 5.5 mM KCl, 4.5 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, pH 7.4). Islets were extensively washed in sterile Hanks solution and cultured in RPMI 1640 medium supplemented with 2 g/l $NaHCO_3$, 1% (v/v) penicillin/streptomycin, 5.6 mM D-glucose and 2% Fetal Bovine Serum, pH 7.4. Approximately 1000 islets/dish were maintained at 37°C in a humidified atmosphere with 3% CO_2 for 3 days in the presence or absence of 1 nM CNTF, and absence or presence of 200 nM Wortmannin (PI3K pathway inhibitor), 50 μM 2'-amino-3'-methoxy-flavone (PD98059) (MAPK pathway inhibitor), 50 μM Curcumin (JAK-STAT3 pathway inhibitor) or 1 μg/ml AG490 (JAK2 inhibitor). The Curcumin dose used was determined by a dose-response curve to find maximal inhibition without completely abolishing STAT3 phosphorylation. The AG490 dose was used according to previous reports [45], and its efficiency was determined by STAT3 phosphorylation (Fig 3A). The medium was renewed every 24 h. Islet experimental groups were assigned according to culture conditions: CTL (Control Group, islets cultured without CNTF) and CNTF (Treated Group, islets cultured in the presence of CNTF).

Insulin Secretion

Batches of 10 islets each were incubated in Krebs-Hepes buffered saline (KHBS, in mM: 115 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 15 Hepes) containing 0.5 g/l BSA and 5.6 mM glucose, pH 7.4, and equilibrated with 95% O_2 and 5% CO_2 for 30 min in 37^{o}C. The medium was

discarded and the islets incubated for a further period of 1 h in 1 ml of KHBS containing 2.8 or 16.7 mM glucose. The supernatant was collected and insulin was measured by radioimmunoassay.

RT-PCR

Groups of 1000 islets were homogenized in Trizol following phenol-chloroform RNA extraction, according to the manufacturer's instructions. RNA integrity was asserted through agarose gel. The reverse transcriptase reaction was performed using 3 µg of total RNA. The reactions were incubated for 5 min at 65^{o}C before the addition of 150 ng random primers, for 10 min at 25^{o}C before addition of 14.3 mM $MgCl_2$, 2.8 mM DTT, 0.4 U/µl RNase-out and at 42^{o}C for 2 min before addition of 1.25 U/µl RNA Super Script II. Samples were incubated at 42^{o}C for 50 min, at 70^{o}C for 15 min, and then cooled to 4^{o}C. The cDNAs obtained were diluted in PCR buffer (60 mM Tris-HCl, 1.5 mM $MgCl_2$, 15 mM NH_4SO_4, pH 10) with 50 mM $MgCl_2$, 0.3 mM each of dATP, dCTP, dGTP and dTTP, 2.5 U/µl Taq DNA polymerase (Gibco/BRL), 10 mM forward primer and reverse primer were then added. PCR amplification of cDNA was performed with a GeneAmp PCR System 9700 (Applied Biosystems). The PCR program employed the following cycle profile: denaturation for 1 min at 94°C, annealing for 1 min, extension for 1.5 min at 72°C, and maximization of strand completion for 7 min at 72°C. Following amplification, the cDNA fragments were analyzed on 1.6 % agarose gels containing a 100 bp DNA molecular weight ladder (Gibco/BRL). PCR products were analyzed by ethidium bromide UV fluorescence in a Gel Doc EQ analyzer (Bio-Rad). Primers were designed and tested against *Rattus norvergicus* genome (Gene Bank) to ensure no amplification of other cDNAs. The Sense (S) and Anti-sense (AS) oligonucleotide primers used were as follows:

AKT2: (S)5' CCTCAAGTACTCATTCCAGAC3'(AS)5'CTCATACACATCTTGCCACAC 3' TM 57ºC - Cycles 30 - NCBI # NM033230

ERK1: (S)5' TCACATCCTGGGTATTCTTGG 3'(AS)5' GGCTCATCACTTGGGTCATA 3' TM 60ºC - Cycles 31 - NCBI#NM 053842

STAT3: (S)5'CACAACCTGCGAAGAATCAAG 3' (AS)5' TCTGAACAGATCCACGATCCT 3' TM 57ºC - Cycles 30 - NCBI# NM012747

SOCS3: (S)5' GGACCAAGAACCTACGCATC 3' (AS)5'TACACAGTCAAAGCGGGGCA 3' TM 59ºC - Cycles 32 - NCBI# NM053565

RPS-29: (S)5'AGGCAAGATGGGTCACCAGC3'(AS)5'AGTCGAATCCATTCAGGTCG3' TM 57ºC - Cycles 27 - NCBI# NM012876

All annealing temperatures and number of cycles were chosen to afford maximal sensibility to the sample cDNA content.

Western-Blot

For basal and phosphorylated protein levels of AKT or ERK 1/2, groups of islets were pelleted by centrifugation and then re-suspended in 50-100 µl of homogenization buffer containing protease inhibitors. To analyze the nuclear translocation of pSTAT3, nuclei from islets treated with or without 50 µM Curcumin (as determined by a dose-response curve; Fig 3 B), in the presence or absence of 1nM CNTF, were isolated by the 1.8 M Sucrose Cushion Buffer Method. The islets or isolated nuclei were sonicated (15 s) and the protein was determined by the Bradford method using bovine serum albumin as standard. The sample volume was adjusted to provide the same amount of protein added to each lane. Samples containing 70 µg of protein from each experimental group were separated by SDS-PAGE, transferred to nitrocellulose membranes and stained with Ponceau. No differences in the total amount of protein were observed, as judged by densitometric analysis of the stained membranes (not shown). Detection was performed using enhanced chemiluminescence (SuperSignal West Pico, Pierce) after incubation with a horseradish peroxidase-conjugated secondary

antibody. Band intensities were quantified by optical densitometry (Scion Image) of the developed autoradiogram.

Cytokine-Induced Apoptosis

For this purpose, neonatal islets were isolated as described and cultured for 48 hours in the presence or absence of 1nM CNTF plus 50 μM of Curcumin or 1ng/ml of AG490, followed by another 24 hours of culture with 10ng/ml of IL-1β. Apoptosis was evaluated by detecting cleaved Caspase 3 by Western-Blot (as described) and DNA Fragmentation (as follows).

DNA fragmentation assay

DNA was isolated from neonatal rat islets, separated in fragmented and integral subunits by trizol/triton method. Both were quantified by the Sybr-green method, as ng/ml of DNA. Data are expressed as Fragmented/Total DNA percentage.

Statistics

Point-to-point comparisons were made by Students t-test. Groups were compared by two-way ANOVA using the unpaired Tukey-Kramer method as post-test. Results were considered significantly different if $P<0.05$. In RT-PCR experiments, results were considered different only if $P<0.001$.

RESULTS

1, AKT and STAT3 mRNA levels in CNTF-treated islets. CNTF treatment for 3 days did not alter mRNA levels of MAPK1 (Fig. 1A), AKT (Fig. 1B) and STAT3 (Fig.1C), compared with Control.

*ERK1/2 and AKT phosphorylation in CNTF-treated islets***.** Since the amount of MAPK and AKT mRNA was not affected by CNTF, we determined whether the CNTF treatment would alter the phosphorylation of these proteins. No differences in the phosphorylated levels of ERK1/2 (Fig. 2A) and

AKT (Fig. 2B) were observed between Control and CNTF-treated islets. Control and CNTF-treated islets, cultured in the presence of 50 μM PD98059 or 200 nM Wortmannin, showed reduced phosphorylated ERK1/2 and AKT, respectively, compared to their own control.

STAT3 phosphorylation in CNTF-treated islets. The activation of the STAT3 pathway involves phosphorylation and dimerization of STAT3, translocation of the STAT3 homodimer to the nucleus and finally binding to and activation of target-gene expression. For this reason, we initially evaluated STAT3 phosphorylation in the cytoplasm and in the nucleus. We found that CNTF promoted an increase (more than three-fold) in phosphorylated STAT3 in both cytoplasm (Fig. 3C) and nucleus (Fig. 3D), and that these effects were blocked by AG490 (a JAK inhibitor) and by Curcumin (a STAT3 inhibitor), indicating that CNTF signals through the JAK/STAT3 pathway in pancreatic islets.

*SOCS-3 expression in CNTF-treated islets***.** To better understand the CNTF signaling in pancreatic islets, we analyzed its effects on SOCS3 expression, a target gene activated by the phosphorylated STAT3. The mRNA levels of SOCS3 were more than 3-fold higher in CNTF-treated islets (Fig. 4A), and this increase was accompanied by a nearly six-fold increase in SOCS3 protein levels (Fig. 4B). The effects of CNTF on SOCS3 mRNA (Fig. 4A) and protein (Fig. 4B) expression were almost blocked by Curcumin, providing further evidence that CNTF signals through the STAT3 pathway in pancreatic islets.

Effects of CNTF on insulin secretion. Islets cultured for 3 days with or without 1nM CNTF, and in the absence or presence of the following inhibitors: 10 μM PD98059 (MAPK inhibitor), 200 nM Wortmannin (PI3K inhibitor), 50 uM Curcumin (JAK/STAT3 inhibitor) were submitted to a glucose challenge (2.8 or 16.7 mM) for 1 h. CNTF impaired GSIS in Control (Fig. 5A), in PD98059-treated (Fig. 5B), and in Wortmannin-treated islets (Fig. 5C). However, the inhibitory effect of CNTF in Curcumin (Fig. 5D), and in AG490-treated islets (Fig. 5E) was significantly lower, confirming that CNTF signals through JAK/STAT3 pathway in pancreatic islets. All results were normalized by the total insulin content.

Effects of CNTF on pancreatic islets survival. Islets cultured for 3 days in the presence of 1nM CNTF showed lower apoptosis compared with Control, as judged by the lower amount of cleaved caspase 3 (Fig 6) and lower DNA fragmentation (Fig 7). Moreover, this CNTF survival effect was inhibited by the STAT3 inhibitor, Curcumin (Fig. 6A and Fig7A), and by the JAK 2 inhibitor, AG490 (Fig. 6B and 7B).

DISCUSSION

CNTF acts as a differentiation and/or survival factor in many cell types [8, 10, 11, 12], and has also been suggested to be an anti-diabetogenic peptide, since it improves peripheral sensitivity to insulin and reduces insulin secretion and obesity [19, 20, 21]. All these beneficial effects make CNTF a candidate to assist in the prevention and/or treatment of diabetes [25]. However, it is still a matter of debate as to whether the observed alterations result from the control of the Central Nervous System via NPY inhibition, a direct action on peripheral tissues or an integration of both. Although these results are somehow related to glucose homeostasis, it has only recently been demonstrated that CNTF has no effect on islet differentiation; instead, CNTF acts mainly as survival factor trough BCL-2 activation [18].

The JAK/STAT3 pathway is the main signaling cascade used by CNTF in many cell types [21, 27, 28], however, CNTF can also signal through MAPK [30] and/or PI3K [24, 29], and the signaling pathway used by CNTF in pancreatic islets is still unknown. The crucial checkpoints of the JAK/STAT3 pathway, in the order for it to occur, are the phosphorylation of inactive STAT3, dimerization, translocation of activated STAT3 to the nucleus, binding to and activation of target genes and, finally, induction of metabolic and/or survival effects. In order to assess whether CNTF signals through the STAT3 pathway, we evaluated all the aforementioned checkpoints and its effects on GSIS and cell survival. The results indicated that CNTF increases STAT3 phosphorylation in the cytoplasm (Fig. 3C), its translocation to the nucleus (Fig. 3D), and enhances mRNA (Fig. 4A) and protein (Fig.

4B) expressions of SOCS3. In addition, CNTF impairs GSIS (Fig. 5) and protects pancreatic islets against cytokine-induced apoptosis, increasing its survival (Fig. 6 and 7). The inhibition of STAT3 by Curcumin blocked all the observed effects (Figs. 3C, 3D, 4A, 4B, 5D, 6A, and 7A). We also observed that JAK inhibition by AG490 partially restores GSIS in the presence of CNTF (Fig 5E) and reduces the CNTF-induced pancreatic islets survival (Figs 6B and 7B). Curcumin may affect other signaling pathways, however our results showed that this drug inhibited STAT3 phosphorylation in pancreatic islets in a dose-dependent manner (Fig 3B), indicating that the effect of Curcumin occurs mainly through STAT3 inhibition. Taken together, these results strongly suggest that CNTF signals through the JAK/STAT3 pathway in neonatal rat islets.

Since STAT3 is not the only pathway used by CNTF, and the MAPK [30] and PI3K cascades [24, 29] are of relevance in some tissues, we verified whether these pathways were also used by CNTF in pancreatic islets. CNTF had no effect on ERK1/2 (Fig. 2A) and AKT (Fig. 2B) phosphorylation, and the blockade of MAPK and PI3K pathways by PD98059 and Wortmannin, respectively (Figs. 5B and 5C), did not alter CNTF-induced impairment of GSIS, implying that MAPK and/or PI3K cascades are not involved in CNTF signaling in rat pancreatic islets. Since gp130 signaling molecules are usually associated with activation of these pathways, our results suggest an unusual pattern of CNTF signaling on pancreatic islets. The reasons for this particular effect are not the focus of the present work, which should be addressed in a future study. Nevertheless, the results obtained with pancreatic islets are in accordance, and corroborate previous findings in numerous other cell types, that CNTF acts mainly through the STAT3 pathway [21, 27, 28]. STAT3 activation promotes cell survival [31, 32] similarly to the effect of CNTF on pancreatic islets [18]. However, the reason as to why CNTF does not activate Akt and/or PI3K as well as the mechanism by which CNTF impairs GSIS remains unknown.

Since CNTF increases SOCS3 expression (Fig. 4), it is possible that CNTF impairs GSIS through SOCS3. This assumption is based on the fact that this protein is activated by STAT3 [47] and

has several biological effects including protection and survival of pancreatic islets [37, 38, 39], regulation of β-cell mass and proliferation [40] and, finally, reduction of insulin secretion [42].

There is evidence that SOCS3 activation by STAT3 protects cells against multiple low doses of STZ-induced type 1 diabetes [39], it has been proposed that a cytokine that activates the STAT3-SOCS3 pathway in pancreatic islets, promoting its survival, could be a potential therapeutic tool for prevention and/or treatment of type 1 diabetes [39]. In this study, we have accumulated evidence that CNTF could be this key cytokine.

In conclusion, CNTF acts on pancreatic islets, activating the JAK/STAT3 pathway, but not the MAPK and PI3K cascades, in turn increasing SOCS3 expression, impairing GSIS and protecting pancreatic islets against cytokine-induced apoptosis, increasing their survival. These findings demonstrate a novel cytokine pathway, the CNTF-STAT3-SOCS3, and suggest that CNTF may be a potential therapeutic tool for the prevention and/or treatment of Type 1 and/or Type 2 Diabetes.

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Figure 1

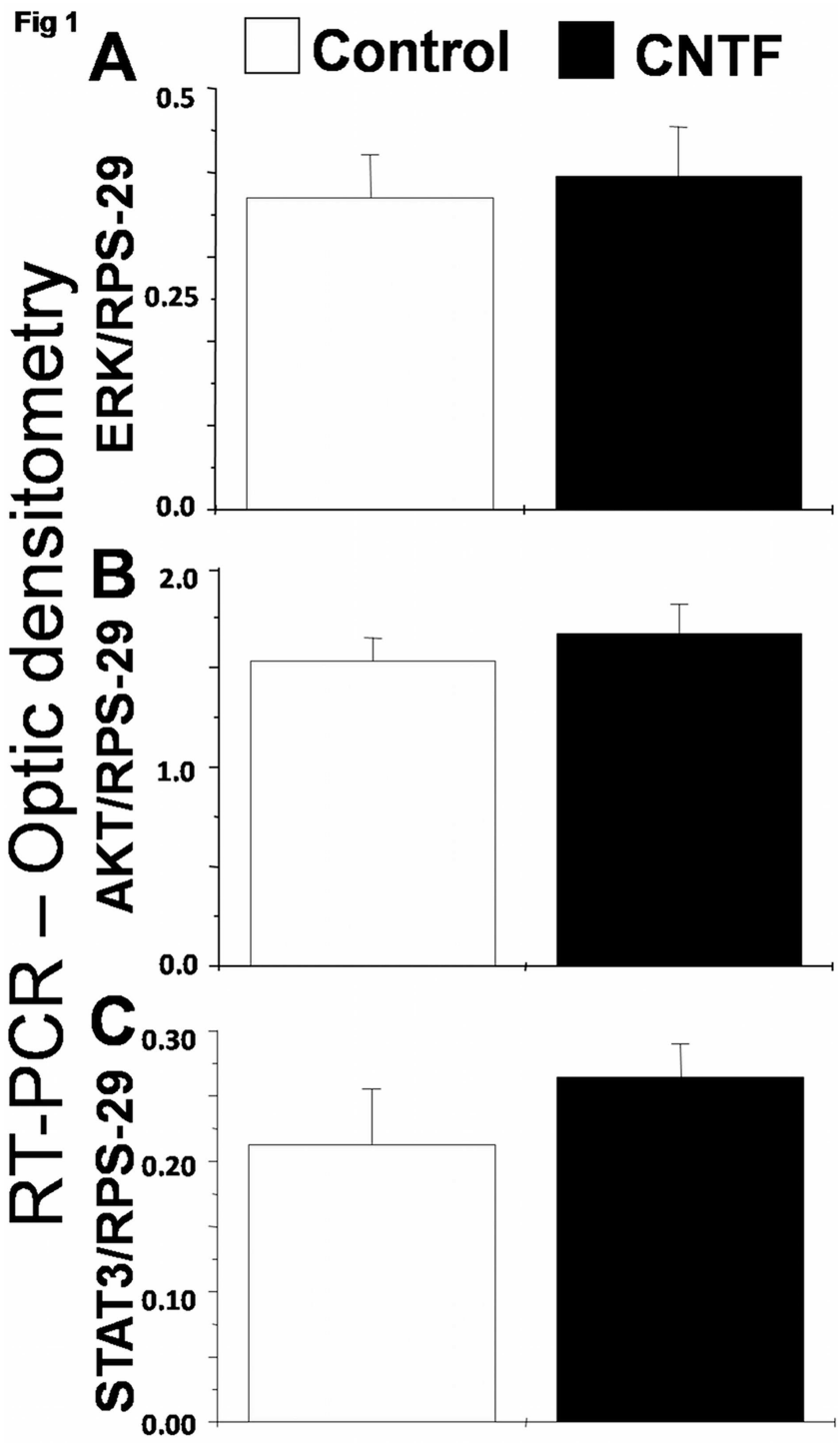

Figure 1. Effect of culture with 1nM CNTF for 3 d on mRNA levels, as evaluated by RT-PCR, of ERK1 (A), AKT (B) and STAT3 (C). RPS-29 was used as internal control, showing no variation amongst the conditions tested. Columns are means ±SEM of 16 experiments.

Figure 2

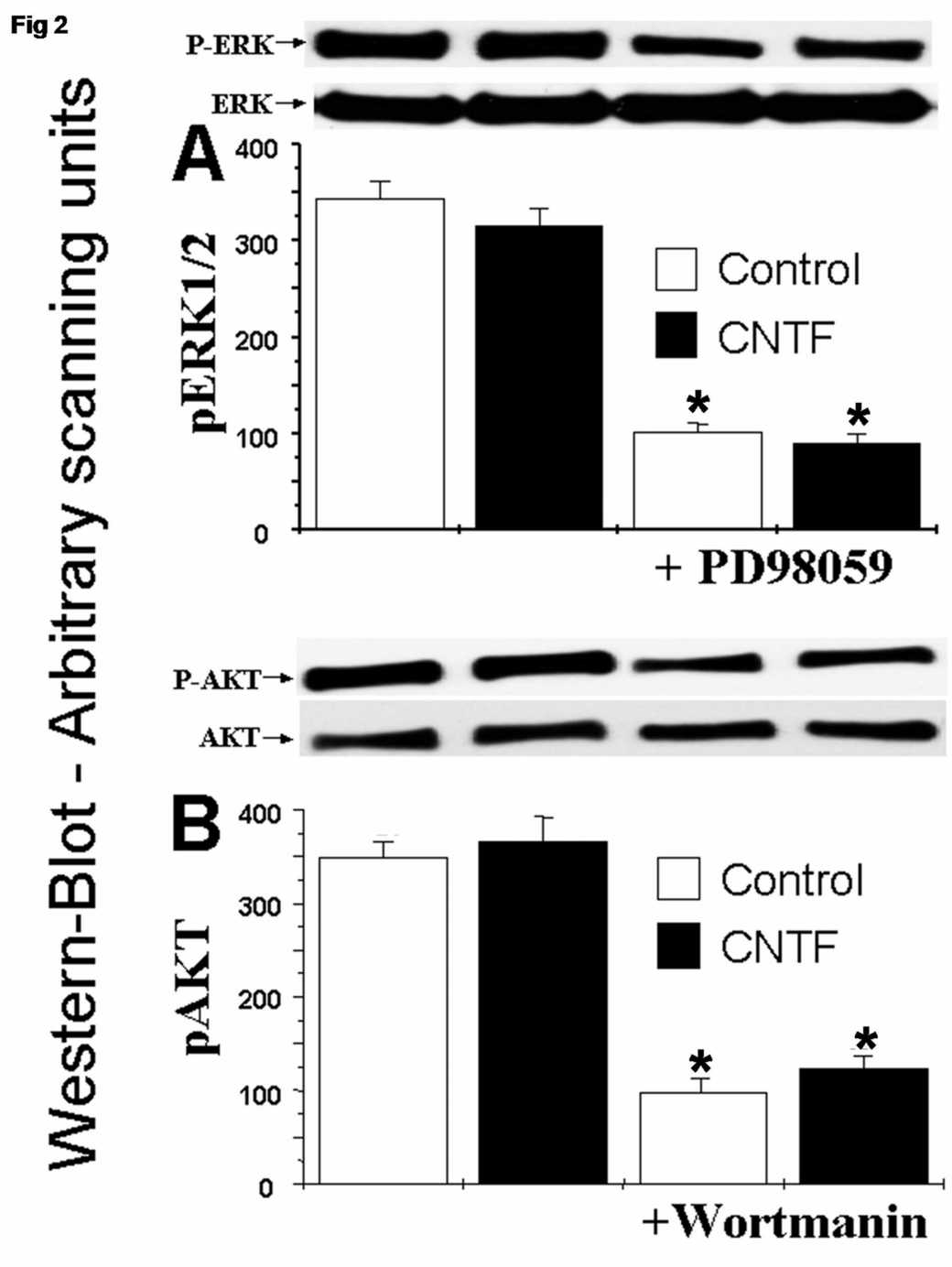

Figure 2. Protein levels of phosphorylated ERK1/2 (A) and phosphorylated AKT (B) in neonate rat islets cultured for 3 d in the presence (CNTF■) or absence (Control □) of 1nM CNTF, plus 10 μM PD98059 or 200 nM Wortmannin. The values are the means ±SEM of six independent experiments. * P<0.05

Figure 3

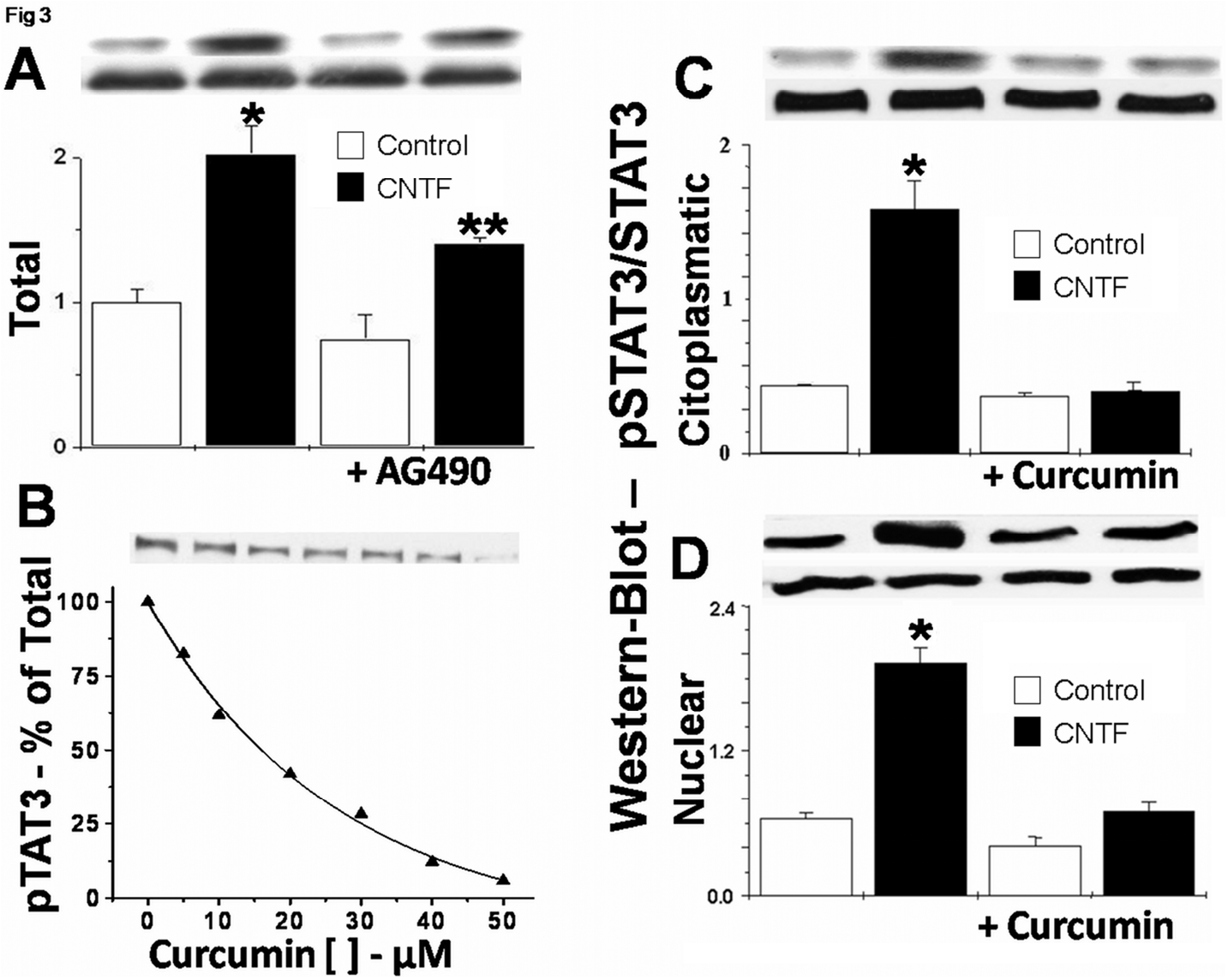

Figure 3. Protein levels of phosphorylated STAT3 of neonate rat islets cultured for 3 d in the presence (CNTF■) or absence (Control □) of 1nM CNTF, plus 1µM AG490 (A) or 50µM of Curcumin, in the Cytoplasmatic (C) and Nuclear (D) compartments. Curcumin dose used was determined by a dose-response curve (B) as evaluated by Western Blot. The values are the means ±SEM of six independent experiments. * $P<0.05$

Figure 4

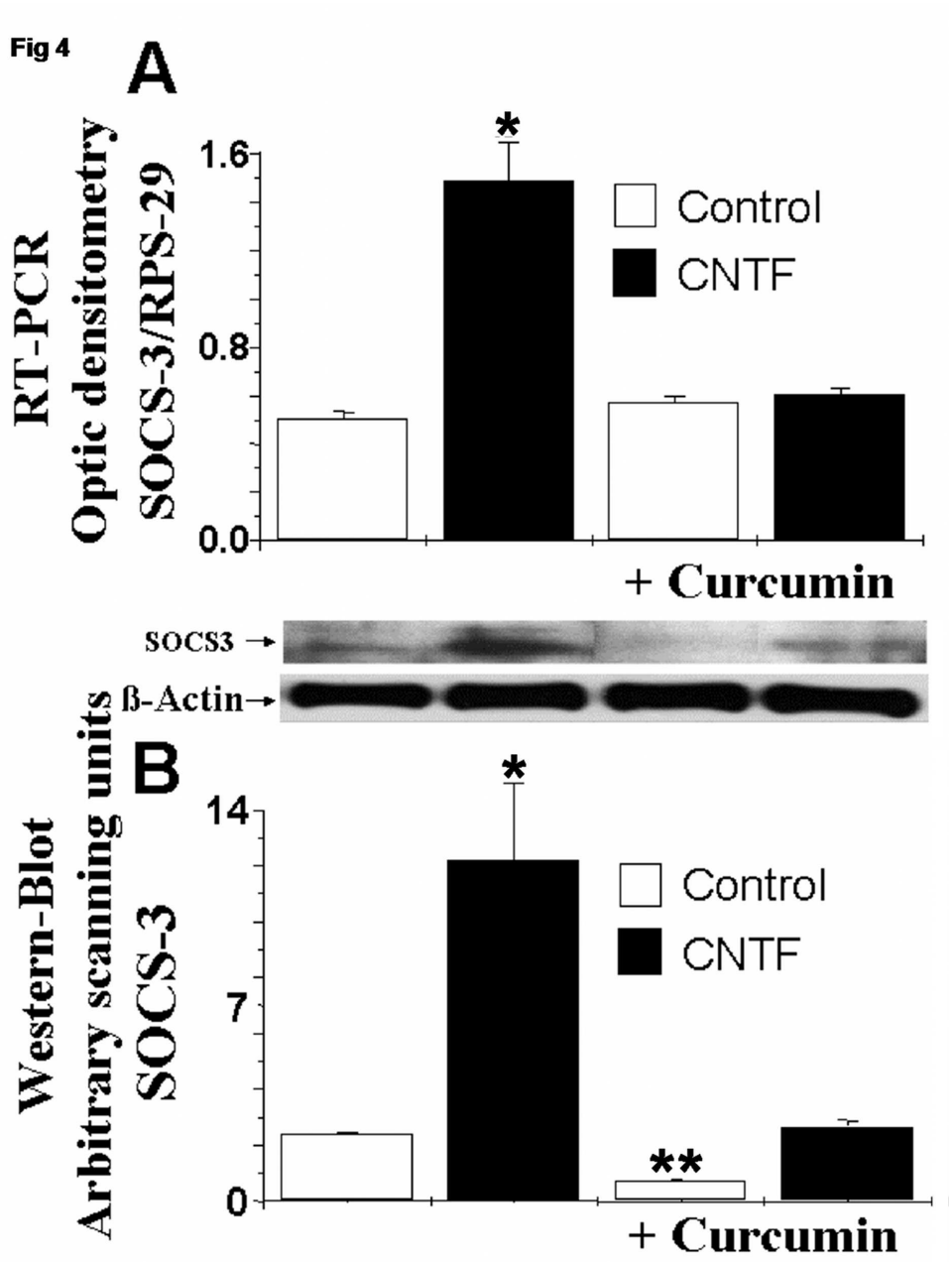

Figure 4. Levels of SOCS3 mRNA (A) and protein (B) expression in neonate rat islets cultured for 3 d in the presence (CNTF■) or absence (Control □) of 1nM CNTF, plus 50 µM Curcumin. The mRNA was evaluated by RT-PCR, using RPS-29 as an internal control, and protein was evaluated by Western blot. Columns are means ±SEM of 16 experiments for mRNA and 6 experiments for protein. * P<0.001.

Figure 5

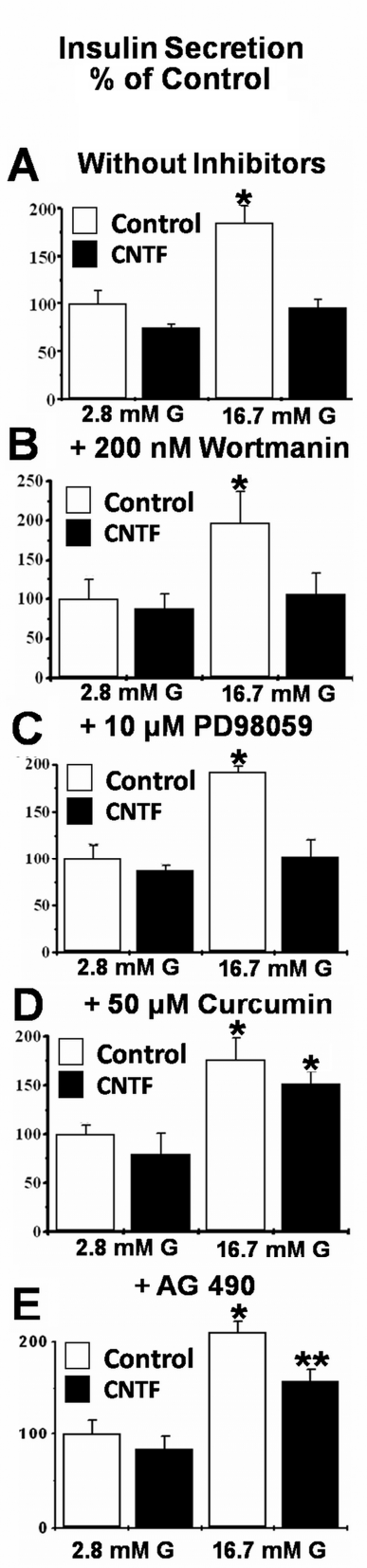

Figure 5. Glucose Stimulated Insulin Secretion (GSIS) in islets cultured for 3d in the presence (CNTF■) or absence (Control □) of 1nM CNTF, plus one of the following inhibitors: None (A), 10 µM PD98059 (B), 200 nM Wortmannin (C), 50 µM Curcumin (D) or 1µM AG490. Islets were pre-incubated for 30 min in KHBS with 5.6 mM glucose, as described, and then incubated either with 2.8 or 16.7 mM glucose for 1 h. Bars are means ±SEM of 8 independent experiments. * $P < 0.05$, **$P < 0.05$.

Figure 6

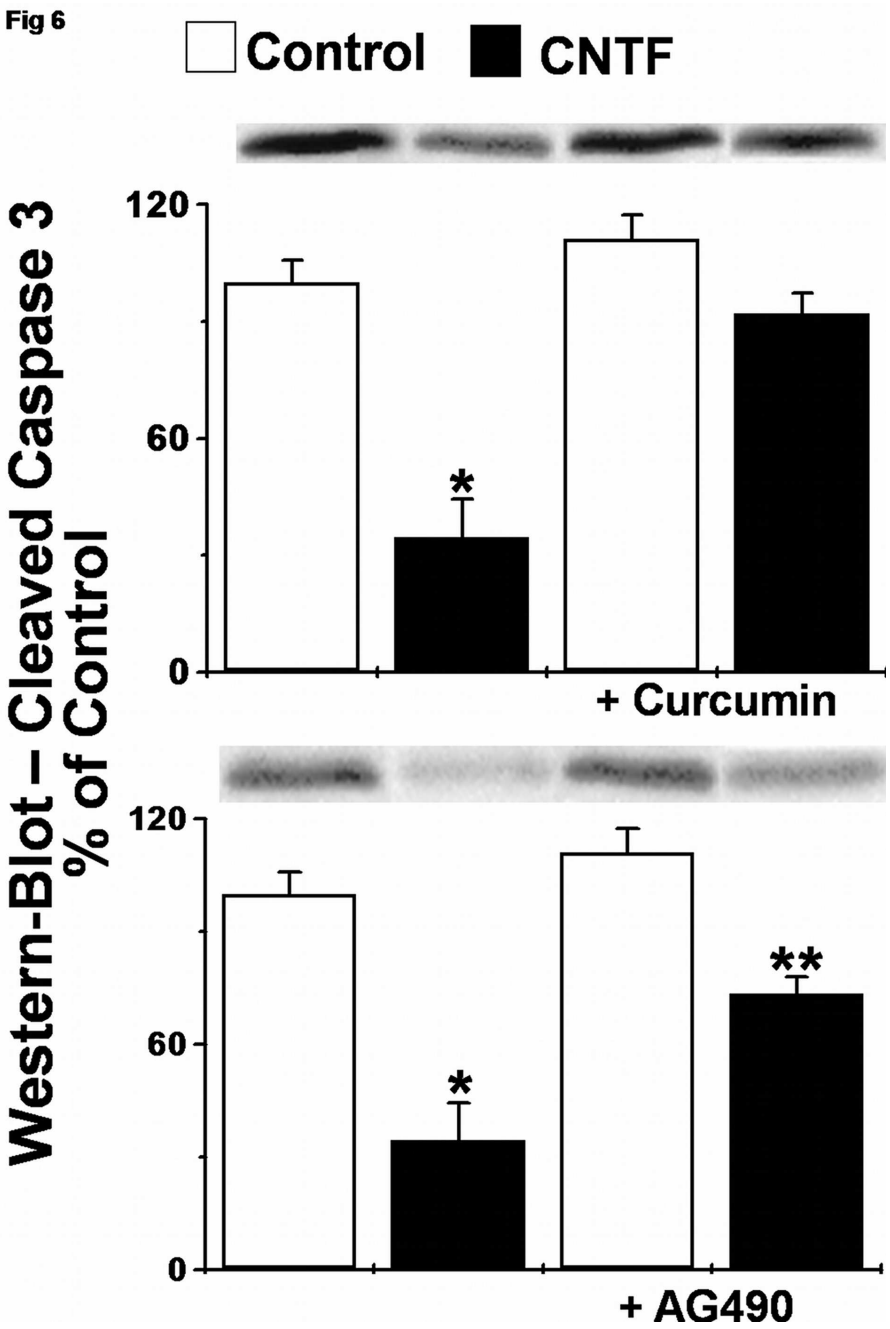

Figure 6. Caspase 3 cleavage was assessed by Western Blotting of cleaved Caspase 3 (Cell-Signalling). Neonatal islets were cultured for 48 hours in the presence or absence of 1nM CNTF plus 50 μM of Curcumin (A) or 1ng/ml of AG490 (B), followed by another 24 hours of culture with 10ng/ml of IL-1β. Data are expressed as cleaved Caspase 3/Total Caspase 3, in % of Control. * $P < 0.05$.

Figure 7

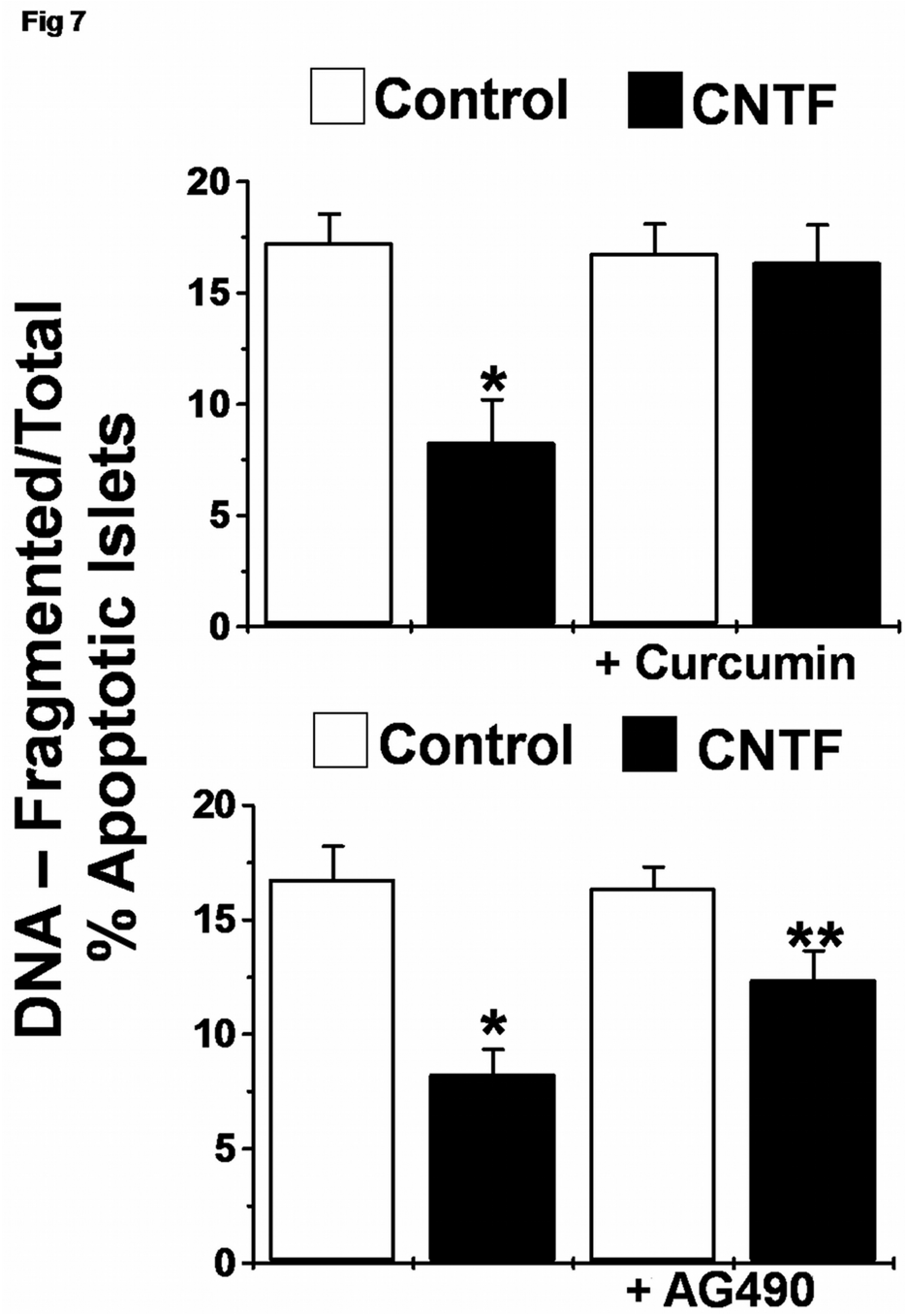

Figure 7. To evaluate DNA fragmentation, neonatal islets were cultured for 48 hours in the presence or absence of 1nM CNTF plus 50 μM of Curcumin (A) or 1ng/ml of AG490 (B), followed by another 24 hours of culture with 10ng/ml of IL-1β. Data are expressed as Fragmented/Total DNA percentage Fragmentation. * $P < 0.05$.

RESUMO PUBLICADO - FESBE 2008

CILIARY NEUROTROPHIC FACTOR (CNTF) AND ITS RECEPTOR, CNTFR-ALPHA, ARE DIFFERENTIALLY EXPRESSED IN RAT PANCREATIC ISLETS THROUGHOUT THE ANIMAL LIFE

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DATA E LOCAL DA APRESENTAÇÃO NO FORMATO PAINEL

21.047 - CILIARY NEUROTROPHIC FACTOR (CNTF) AND ITS RECEPTOR, CNTFR-ALPHA, ARE DIFFERENTIALLY EXPRESSED IN RAT PANCREATIC ISLETS THROUGHOUT THE ANIMAL LIFE.

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Endocrinologia

Data: 22/8/2008 **Horário:** 08h00 às 10h00 **Local:** Salão Imperial

PUBLICAÇÃO DO RESUMO NOS ANAIS DO CONGRESSO FESBE 2008: # 21.047, Página 89.

ABSTRACT

Objectives: CNTF is a cytokine that promotes pancreatic islets survival. The Peptide does not contain an exocytosis sequence, and therefore is not secreted in response to any stimuli but released as a consequence of direct injury to the cell. To exert its actions, it requires a specific receptor, CNTFR-alpha, associated to gp130 and LIF receptor. The CNTF effects on many organisms are related to the animal life stage, corresponding to a differential expression of CNTF as well as CNTFR-alpha. The aim of this study was to assess how CNTF and its specific receptor CNTFR-alpha are expressed in rat pancreatic islets throughout the animal life.
Methods and Results: Pancreatic islets were isolated by the colagenase method from rats in the following ages: Neonatal (2 days-old), young (2 months), mature (6 months) and old (20 months). We verified CNTF and CNTFR-alpha mRNA levels by RT-PCR and protein levels by Western-Blot. Data are means ±se. $p<0,05$ Our results showed that CNTF mRNA and protein levels were around two-fold higher in pancreatic islets of 2, 6 and 20 months-old rats when compared to neonatal rats. The same results were obtained for CNTF receptor alpha mRNA and protein levels.
Conclusion: CNTF as well as its specific receptor, CNTFR-alpha is differentially expressed in rat pancreatic islets throughout the animal life, both being more prominent on the mature animals.

INTRODUÇÃO

O CNTF em si não possui seqüência sinal de exocitose, mas supõe-se que exerça seus efeitos após ser liberado das células por algum mecanismo induzido por dano.

Curiosamente, animais knockout para o gene do CNTF se desenvolvem normalmente. Somente na fase adulta os animais começam a apresentar perda de neurônios motores com decorrente

fraqueza muscular, o que sugere que o CNTF não seja essencial durante o desenvolvimento, mas sim como protetor em situações de estresse que possam levar à morte celular.

O peptídio sinaliza utilizando um complexo receptor que guarda diversas semelhanças e compartilha subunidades com os complexos receptores de interleucina-6 (IL-6) e leukemia inhibitory factor (LIF). A subunidade α do complexo receptor (CNTFRα) confere especificidade ao CNTF, e é extremamente bem conservada entre as espécies. O CNTFRα não possui domínio transmembrana convencional, e supostamente é ancorado à membrana celular via ligação à glicoproteínas.

Essas evidências sugerem que as expressões diferenciadas tanto do CNTF quanto de seu receptor específico ao longo da vida animal (*time-course*) sejam aspectos fundamentais de suas funções. O *time-course* dessas proteínas em ilhotas de ratos ainda não foi investigado, visto que mesmo os efeitos do CNTF sobre as células-β ainda não são totalmente conhecidos.

Dessa forma, nosso objetivo foi a investigação do *time-course* do CNTF e de seu receptor CNTFRα em ilhotas pancreáticas de ratos com 1-2 dias de vida (neonatos), com 2 meses de vida (jovens), 6 meses (adultos) e 20 meses (idosos), através da análise das expressões gênica e protéica das referidas proteínas.

MATERIAL E MÉTODOS

Animais: Foram utilizados ratos Wistar neonatos (1-2 dias de vida) e ratos jovens (60 dias) provenientes do biotério central da UNICAMP. Alguns animais jovens foram mantidos em biotério controlado do Departamento de Fisiologia e Biofísica – Instituto de Biologia – UNICAMP até que atingissem a idade adulta (6 meses) ou idosa (20 meses).

Cultura e isolamento de ilhotas - todo o material utilizado para cultura de ilhotas foi submetido a condições especiais de esterilização. Para cada experimento foram utilizados aproximadamente 40-60 ratos neonatos. Após decapitação e assepsia da região abdominal, os animais foram colocados em placas de Petri de vidro e levados ao fluxo laminar (VECO, HLFS-12) para a retirada dos pâncreas. Durante a cirurgia todos os pâncreas foram mantidos em solução de Hanks. A seguir, lavados, tratados com colagenase (0.2 mg/órgão) e transferidos para um tubo de ensaio de 15 mL com tampa, agitado durante 5 min em banho-maria a 37ºC. O material foi lavado 3 vezes com solução de Hanks para eliminação da colagenase, cada lavagem seguida de centrifugação e descarte do sobrenadante. Uma 4º lavagem foi realizada com meio de cultura (RPMI 1640 + 2 g/L NaHCO3 + 5% soro fetal bovino + 2 µg/mL penicilina/estreptomicina + 5.6 mM glicose). O tecido pancreático parcialmente digerido foi transferido para placas de cultura (Falcon 3003 – 100 x 120 mm) contendo 10 ml do meio de cultura suplementado com ou sem 1nM de CNTF teste. As placas foram mantidas em incubadora de com atmosfera úmida de 5% CO_2 (CO-24, New Brunswick Scientific Co. Inc., New Jersey, USA) a 37ºC.

Identificação e quantificação protéica por imunobloting - As proteínas estudadas neste trabalho foram avaliadas pela técnica de Western blot.

RT-PCR - A reação da transcriptase reversa será realizada a 42 ºC durante 50 min, em 20 µL de uma mistura contendo 2,0 µg de RNA total + 10mM de dNTP mix + 40 U/µl de inibidor de Rnase + 0,1M de DTT + 5X first-strand buffer + 0,5 µg/ml de oligo dT + 200 U da enzima Super Script II RNase H Reverse Transcriptase (GIBCO BRL – Life Technologies).

O cDNA foi amplificado com os "primers": sense e antisense correspondentes ao CNTF e CNTFRα. A reação PCR foi realizada em 25 µL de uma mistura contendo tampão da Taq polimerase + 50 mM de $MgCl_2$ + 10 mM de cada deoxinucleosídeo trifosfato, 2,5 U/µl de Taq DNA polimerase + 10 pmol de "primer" sense + 10 pmol de "primer" antisense + cDNA. Alíquotas de 5 µL do produto da PCR foram

analisados por eletroforese em gel de agarose 1,0% preparado em tampão TBE. Após coloração com brometo de etídio (0,5 μg/mL), o gel foi fotografado sob luz UV e quantificado em aparelho Eagle Eye.

RESULTADOS

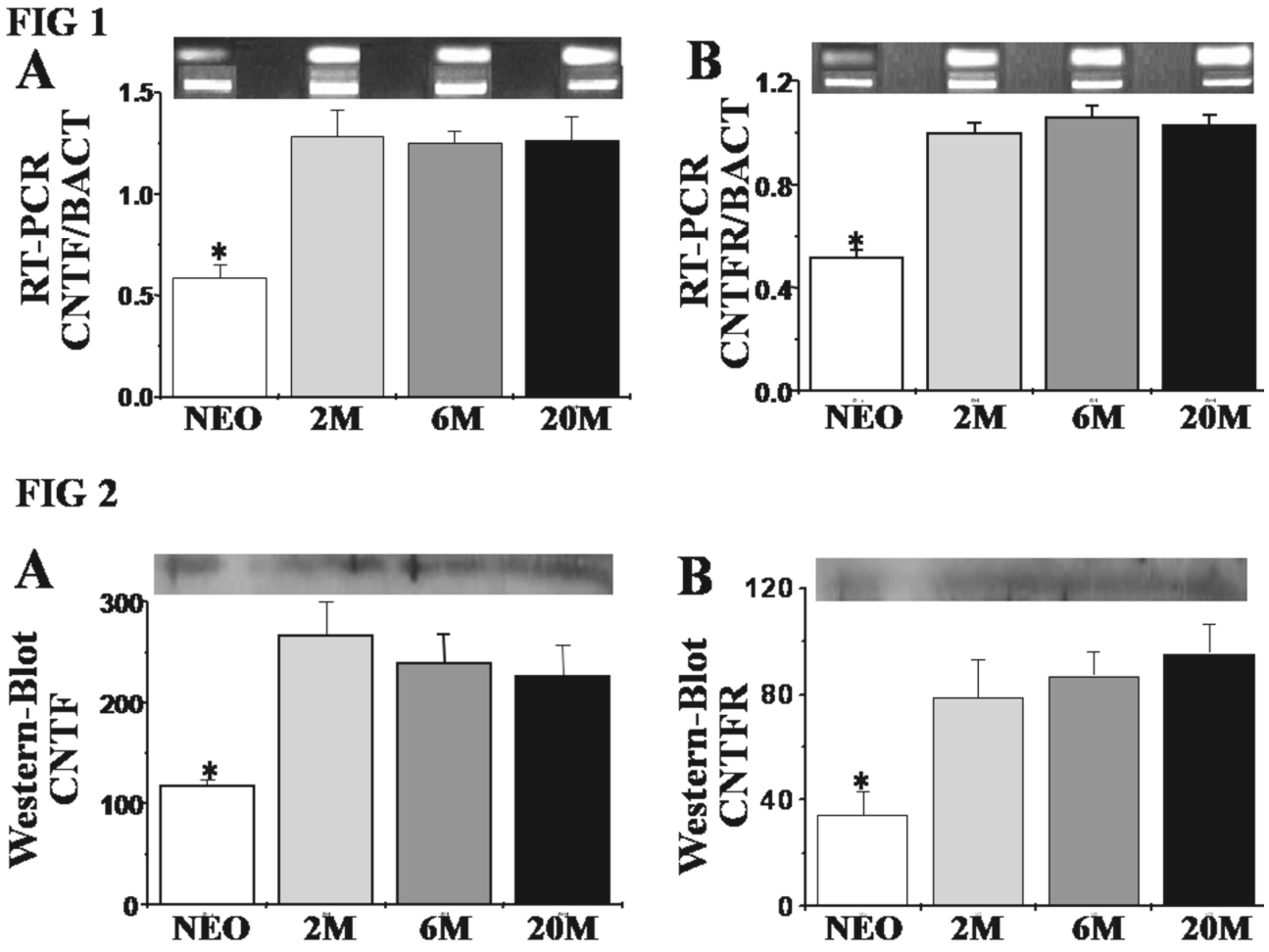
Observamos que os níveis de mRNA (Fig. 1A) e proteína (Fig 2A) de CNTF são equivalentes a 50% dos encontrados em animais de 2, 6 e 20 meses, a exemplo do que ocorre com os níveis de mRNA (Fig 1B) e proteína (Fig 2B) de CNTFRα.

DISCUSSÃO

Os resultados obtidos sugerem, a exemplo do que já é conhecido, que o CNTF é mais relevante em animais de idade mais avançada, sendo pouco importante nos processos de diferenciação e maturação das ilhotas que ocorrem primordialmente nas primeiras fases da vida do animal. Seu papel seria o de proteger as ilhotas contra diversos tipos de estresse, aumentando sua chance de sobrevivência.

Além disso, os resultados estão de acordo com o que já foi descrito em relação à expressão do CNTF em células nervosas.

Figuras



Figures 1-2. CNTF and CNTFR expressions in pancreatic islets were evaluated in neonatal (NEO), 2 months-old (2M), 6 months-old (6M) and 20 months-old (20M) rats. CNTF (Fig 1A) and CNTFR (Fig 1B) mRNa levels as evaluated by rt-PCR, n=16. *P < 0.001. CNTF (Fig 2A) and CNTFR (Fig 2B) as evaluated by Western Blotting, data expressed as Optic Densitometry, N=4. *P < 0.05.

CONSIDERAÇÔES FINAIS

Ilhotas pancreáticas expressam receptores para diversos fatores de crescimento nervoso (NGFs) e podem ter uma resposta neurotípica a diversos desses fatores [20] e, sendo assim, o sistema nervoso associado a essas ilhotas tem efeito importante na modulação de sua função, atividade e sobrevivência. Mais ainda, as próprias ilhotas expressam diversas citocinas e fatores neuronais, com função autócrina e parácrina. A investigação dos efeitos dessas citocinas e fatores neuronais em ilhotas pancreáticas, bem como sua expressão pelas mesmas, constituem um amplo campo de estudo.

O CNTF, uma dessas citocinas, pertence à família da interleucina–6 (IL-6) e foi primeiramente reconhecido como um fator de sobrevivência neuronal, mais precisamente, de sobrevivência de neurônios ciliares parassimpáticos. Além disso, exerce outros efeitos neurotróficos como fator de sobrevivência de neurônios simpáticos, sensoriais e motores *in vitro* e *in vivo* [9, 10], e fator de diferenciação de células progenitoras de astrócitos em astrócitos tipo 2 [11].

Na última década, e com maior frequência nos últimos anos, diversos trabalhos têm relatado como o CNTF melhora diversos parâmetros metabólicos relacionados ao desenvolvimento e progressão do Diabetes Mellitus Tipo 2, como diminuição da resistência periférica à insulina e aumento do metabolismo de lipídios [22, 24, 25, 26, 27]. Apesar de promissores, esses resultados esbarravam em diversos desafios: Em primeiro lugar, animais tratados com CNTF apresentam severos efeitos colaterais como caquexia, anorexia e não raramente morte, e as causas para esses efeitos não são ainda plenamente conhecidas [27]. Sabe-se que ele age em neurônios do SNS inibindo a expressão e secreção de NPY, o que leva a uma redução do apetite e da ingesta alimentar, e concomitantemente atua nos tecidos periféricos aumentando a lipólise e diminuindo a lipogênese [27]. Essas ações (central e periférica), combinadas levariam ao quadro anoréxico e caquético dos animais. Diversas estratégias têm sido tentadas para evitar estes efeitos, desde diferentes formas de administração até geração de peptídios análogos ao CNTF, algumas delas promissoras, mas nenhuma perfeitamente adequada até o momento, o que nos leva ao segundo desafio: assim como outras

citocinas da família das interleucinas 6, à qual pertence, o CNTF utiliza o complexo receptor gp130 [30], reconhecidamente promíscuo, o que significa que pode ocorrer sinalização cruzada com outras proteínas que sinalizem através desta mesma via, o que dificulta seu estudo in-vivo. No caso específico do CNTF, é conhecido que ocorre transsinalização entre o CNTF e a leptina, com uma substância agindo sobre o receptor da outra, mais comumente o CNTF agindo sobre o receptor de leptina em todos os tecidos estudados até o momento, inclusive superando a resistência à leptina [24, 25], o que contribuiria ainda mais com os efeitos colaterais do CNTF. Dadas estas peculiaridades da ação do CNTF, a investigação de suas vias de sinalização é um ponto importante de seu estudo.

Em terceiro lugar, apesar da quantidade de resultados relativos aos efeitos benéficos do CNTF em animais resistentes à insulina ou mesmo com Diabetes Tipo 2, eles são relativos apenas à sua ação periférica, como tecidos adiposo e muscular, ou no SNC, enquanto seus efeitos sobre o principal órgão regulador da homeostase glicêmica, as ilhotas pancreáticas, permanecem desconhecidos, representando um entrave ao uso terapêutico do peptídio. A primeira e até então única tentativa de análise nesse sentido revelou que acentuava a redução da secreção insulina estimulada por glicose em ilhotas tratadas com IL-1β, concomitante a aumento marginal na produção de NO. [23]

Por último, o CNTF é expresso diferencialmente ao longo da vida do animal de acordo com o tecido pesquisado, o que torna a idade do animal estudado um fator a ser levado em conta nos estudos realizados com essa citocina.

Tendo estes dados em mente, decidimos que seria de grande relevância a investigação de alguns destes pontos em aberto sobre os efeitos do CNTF, e nosso objetivo foi investigar quais os efeitos do CNTF sobre as ilhotas pancreáticas, seu mecanismo de ação e sua expressão nas ilhotas pancreáticas ao longo da vida dos animais.

Quanto ao primeiro ponto, visto que o CNTF age como fator de diferenciação e/ou sobrevivência celular, definimos que o primeiro aspecto a ser investigado seria os efeitos do CNTF sobre a maturação das ilhotas pancreáticas. Para isso, utilizamos ilhotas de ratos com 1 a 2 dias de vida que passam por um processo de maturação quando mantidas em cultura. Essa maturação "in vitro" foi avaliada através análise da secreção de insulina estimulada por glicose, do metabolismo da glicose, do metabolismo mitocondrial e da expressão de proteínas marcadoras de ilhotas pancreáticas maduras, tais como: GLUT2, Insulina, PKCα, PDX-1, NKX6.1 e Glucokinase IV.

Os resultados obtidos mostraram que o CNTF inibe a secreção de insulina estimulada por glicose (Figura 1, Artigo1) e o metabolismo mitocondrial (Figura 2, Artigo1) sem, no entanto, alterar o metabolismo de glicose (Figura 3, Artigo1) e a expressão das proteínas associadas à função das ilhotas (Figuras 4 e 5, Artigo 1), indicando claramente que o CNTF não promovia a diferenciação e/ou a maturação das ilhotas.

Sendo assim, o próximo passo foi investigar os possíveis efeitos do CNTF sobre a sobrevivência dessas ilhotas. O modelo de indução de apoptose usado foi o de redução da concentração de soro fetal bovino no meio de cultura dos usuais 5-10% para apenas 2%. Como critério de avaliação de apoptose, averiguamos a expressão de proteínas relacionadas a esse processo, tanto pró-apoptóticas como BAD e BAX, quanto anti-apoptóticas, como AKT, BCL-2, PAX4 e CX36, além da atividade de Caspase-3 e Fragmentação de DNA.

Os resultados mostraram que o CNTF não exerce efeito sobre BAD e BAX, proteínas apoptóticas, mas aumenta a expressão das anti-apoptóticas BCL-2, PAX4 e CX36 (Figuras 6 e 7, Artigo 1), indicando que o CNTF promove a sobrevivência de ilhotas pancreáticas de ratos neonatos mantidas em cultura. Esses resultados foram reforçados com a observação de que ilhotas tratadas com CNTF apresentaram atividade da Caspase-3 e a fragmentação de DNA reduzidas (Figura 8, Artigo 1). Assim, respondendo a primeira questão, concluímos que o CNTF não promove a diferenciação e/ou

maturação das ilhotas pancreáticas de ratos neonatos, mas sim sua sobrevivência, principalmente pelo aumento da expressão de BCL-2.

O próximo passo e, respondendo a segunda questão, analisamos o mecanismo de ação do CNTF sobre as ilhotas pancreáticas. Tal preocupação se deveu ao fato de que a sinalização pelo complexo receptor gp-130 é bastante complexa levando à ativação de diversas vias, dependendo da célula-alvo e da composição do complexo, sendo que a principais vias reportadamente ativadas pelo CNTF são a da MAPK, da PI3K e da JAK/STAT3. Assim, analisamos quais dessas vias são ativadas pelo peptídeo nas ilhotas neonatais.

Inicialmente, observamos que o CNTF não alterou os níveis de mRNA de STAT3, AKT ou ERK, indicando que não havia modificação na expressão dessas proteínas. (Figura 1, Artigo 2). Verificamos, ainda, que o CNTF não alterou os níveis de fosforilação de AKT e ERK 1/2, tanto na presença quanto na ausência de seus respectivos inibidores (Wortmanina e PD98059), indicando que o CNTF não sinaliza por essas vias em ilhotas pancreáticas (Figura 2, Artigo 2). Contudo, observamos que os níveis de fosforilação de STAT3 estão aumentados em resposta ao CNTF, e que quando inibimos a ativação de JAK (com AG490) ou de STAT3 (com curcumina), o efeito do CNTF é bloqueado (Figura 3, Artigo2), indicando que ele sinaliza via JAK/STAT3. Depois de fosforilação ocorre a dimerização da STAT3 que migra para o núcleo. Esta etapa de translocação de STAT3 fosforilada ao núcleo também é aumentada com CNTF e inibida pela Curcumina (Figura 3D, Artigo2). Após migrar ao núcleo, os dímeros de STAT3 se ligam a regiões específicas do DNA promovendo a ativação ou inativação de genes alvo, e esta etapa foi avaliada através da análise da expressão de SOCS-3 a qual foi aumentada cerca de 7 vezes nas ilhotas tratadas com CNTF. Esse efeito é inibido por curcumina (Figura 4, Artigo2), mais uma vez reforçando que o CNTF sinaliza via JAK/STAT3.

A seguir, investigamos os efeitos dos inibidores das vias da MAPK (PD98059), PI3K (Wortmanina), e JAK (AG490)/STAT3 (Curcumina) sobre a secreção de insulina estimulada por glicose, e os resultados mostraram que a redução da GSIS pelo CNTF só era inibida pelos inibidores de JAK (AG490) e Curcumina (STAT3) (Figura 5, Artigo 2), indicando novamente que esta é a via de sinalização do CNTF em ilhotas pancreáticas.

Por fim, analizamos os efeitos do CNTF sobre a apoptose induzida por citocinas inflamatórias, no caso IL-1β, e se esse efeito seria bloqueado pelos inibidores da via JAK/STAT3. Descobrimos que o CNTF promove a sobrevivência de ilhotas pancreáticas expostas à ação de citcocinas inflamatórias, um modelo semelhante à DM1, e que esse efeito é bloqueado pelos inibidores AG490 e Curcumina (Figuras 6 e 7, Artigo 2).

Com base nos resultados obtidos, podemos concluir que o CNTF sinaliza via JAK/STAT3, mas não através das vias da MAPK e PI3K. Observamos, ainda, que o CNTF inibe a apoptose induzida por citocinas inflamatórias, trazendo informações importantes sobre o mecanismo de ação do CNTF nas ilhotas pancreáticas.

A última questão diz respeito à distribuição do CNTF e seu receptor específico, CNTFR, nas ilhotas pancreáticas de ratos ao longo da vida dos animais. Portanto, analisamos os níveis de mRNA e proteína de ambos, em diversas fases da vida do animal: Recém-nascidos (neonatos), jovens (2 meses), adultos (6 meses) e idosos (20 meses). Os resultados mostraram que tanto o CNTF quanto seu receptor específico têm suas expressões aumentadas a partir de 2 meses, as quais se mantêm constantes até os 20 meses de idade. (Figuras 1 e 2, Resumo FESBE 2008).

CONCLUSÕES

O CNTF não promove a maturação de ilhotas pancreáticas de ratos neonatos, pois não altera seu metabolismo de glicose e a expressão de proteínas-chave para sua função, e, além disso, ainda reduz o seu metabolismo geral e inibe a secreção de insulina estimulada por glicose.

O CNTF protege as ilhotas pancreáticas contra apoptose decorrente de privação de soro, e faz isso aumentando a expressão de proteínas anti-apoptóticas, como PAX4 e, mais notadamente, de BCL-2.

Todos esses efeitos do CNTF em ilhotas pancreáticas são mediados via JAK/STAT3, visto que observamos que ele promove a ativação de JAK, fosforilação de STAT3, translocação de STAT3 ao núcleo, ativação de SOCS-3, um gene-alvo da STAT3 e que tanto a redução na secreção de insulina estimulada por glicose quanto a redução da apoptose induzida por citocina (IL-1β) são bloqueadas por inibidores de JAK (AG490) e de STAT3 (Curcumina).

Outras vias de sinalização potencialmente ativadas por sinalização via gp130 como MAPK e PI3K não são ativadas pelo CNTF, mostrando um singular método de sinalização do CNTF em ilhotas pancreáticas.

Finalmente, o CNTF possui expressão diferenciada em ilhotas pancreáticas de ratos ao longo da vida do animal, sendo inicialmente mais baixa (neonatos) e atingindo a maturidade aos 2 meses de idade, e esses níveis de expressão se mantém constantes por toda a fase adulta (6 meses) e até a idade avançada (20 meses).

O efeito protetor do CNTF contra apoptose de ilhotas pancreáticas de ratos, tanto decorrente da privação de soro quanto induzida por citocina inflamatória (IL-1β) sugere que o CNTF pode ser uma importante ferramenta na prevenção e/ou tratamento do DM1.

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ANEXO 1

1: J Endocrinol. 2007 Oct;195(1):157-65

Ciliary neurotrophic factor promotes survival of neonatal rat islets via the BCL-2 anti-apoptotic pathway.

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Ciliary neurotrophic factor (CNTF) belongs to the cytokine family and increases neuron differentiation and/or survival. Pancreatic islets are richly innervated and express receptors for nerve growth factors (NGFs) and may undergo neurotypic responses. CNTF is found in pancreatic islets and exerts paracrine effects in neighboring cells. The aim of this study was to investigate possible effects of CNTF on neonatal rat pancreatic islet differentiation and/or survival. For this purpose, we isolated pancreatic islets from neonatal rats (1-2 days old) by the collagenase method and cultured for 3 days in RPMI medium with (CNTF) or without (CTL) 1 nM CNTF. Thereafter, glucose-stimulated insulin secretion (RIA), general metabolism by (NAD(P)H production; MTS), glucose metabolism ((14)CO(2) production), gene (RT-PCR), protein expression (western blotting), caspase-3 activity (Asp-Glu-Val-Asp (DEVD)), and apoptosis (DNA fragmentation) were analyzed. Our results showed that CNTF-treated islets demonstrated reduced glucose-induced insulin secretion. CNTF treatment did not affect glucose metabolism, as well as the expression of mRNAs and proteins that are crucial for the secretory process. Conversely, CNTF significantly increased mRNA and protein levels related to cell survival, such as Cx36, PAX4, and BCL-2, reduced caspase-3 activity, and islet cells apoptosis, suggesting that CNTF does not affect islet cell differentiation and, instead, acts as a survival factor reducing apoptosis by increasing the expression of the anti-apoptotic BCL-2 protein and decreasing caspase-3 activity.

PMID: 17911407 [PubMed - indexed for MEDLINE]

ANEXO 2

Elsevier Editorial System(tm) for Cytokine

Manuscript Number: CYTO-08-215R1

Title: CILIARY NEUROTROPHIC FACTOR (CNTF) SIGNALS THROUGH STAT3-SOCS3 PATHWAY AND PROTECTS RAT PANCREATIC ISLETS FROM CYTOKINE-INDUCED APOPTOSIS

Article Type: Regular Article

Keywords: Ciliary Neurotrophic Factor, CNTF; Signal Transducer and Activator of Transcription 3, STAT3; Suppressor of Cytokine Signaling 3, SOCS3; Pancreatic Islets apoptosis; Type 1 and Type 2 Diabetes

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Abstract: CNTF is a cytokine that promotes survival and/or differentiation in many cell types, including rat pancreatic islets. In this work, we studied the mechanism of CNTF signal in neonatal rats pancreatic islets isolated by the collagenase method and cultured for 3 days in RPMI medium without (CTL) or with 1nM of CNTF (CNTF). The medium contained, when necessary, specific inhibitors of the PI3K, MAPK and JAK/STAT3 pathways. mRNA expression (RT-PCR) and protein phosphorylation (Western blot) of Akt, ERK1/2 and STAT3, and SOCS-3 (RT-PCR and Western-blot), as well as glucose-stimulated insulin secretion (GSIS) (Radioimmunoassay), were analyzed. Our results showed that Akt, ERK1 and STAT3 mRNA expression, as well as phosphorylated Akt and ERK1/2, was not affected by CNTF treatment. CNTF increased cytoplasmatic and nuclear phosphorylated STAT3, and the SOCS3 mRNA and protein expression. In addition, CNTF lowered apoptosis and impaired GSIS. These effects were blocked by the JAK inhibitor, AG490 and by the STAT3 inhibitor Curcumin, but not by the MAPK inhibitor, PD98059, nor by the PI3K inhibitor, Wortmannin. In conclusion, CNTF signals through the JAK2/STAT3 cascade, increases SOCS3 expression, impairs GSIS and protects neonatal pancreatic rat islets from cytokine-induced apoptosis. These findings indicate that CNTF may be a potential therapeutic tool against Type 1 and/or Type 2 Diabetes.

ANEXO 3

21.047 - **Ciliary Neurotrophic Factor (CNTF) and its receptor, CNTFR-alpha, are differentially expressed in rat pancreatic islets throughout the animal life.**
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Endocrinologia

Data: 22/8/2008 **Horário:** 08h00 às 10h00 **Local:** Salão Imperial

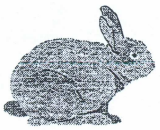
PUBLICAÇÃO DO RESUMO NOS ANAIS DO CONGRESSO FESBE 2008**: #** 21.047, Página 89

ABSTRACT

Objectives: CNTF is a cytokine that promotes pancreatic islets survival. The Peptide does not contain an exocytosis sequence, and therefore is not secreted in response to any stimuli but released as a consequence of direct injury to the cell. To exert its actions, it requires a specific receptor, CNTFR-alpha, associated to gp130 and LIF receptor. The CNTF effects on many organisms are related to the animal life stage, corresponding to a differential expression of CNTF as well as CNTFR-alpha. The aim of this study was to assess how CNTF and its specific receptor CNTFR-alpha are expressed in rat pancreatic islets throughout the animal life.
Methods and Results: Pancreatic islets were isolated by the colagenase method from rats in the following ages: Neonatal (2 days-old), young (2 months), mature (6 months) and old (20 months). We verified CNTF and CNTFR-alpha mRNA levels by RT-PCR and protein levels by Western-Blot. Data are means ±se. $p<0,05$ Our results showed that CNTF mRNA and protein levels were around two-fold higher in pancreatic islets of 2, 6 and 20 months-old rats when compared to neonatal rats. The same results were obtained for CNTF receptor alpha mRNA and protein levels.
Conclusion: CNTF as well as its specific receptor, CNTFR-alpha is differentially expressed in rat pancreatic islets throughout the animal life, both being more prominent on the mature animals.

ANEXO 4

UNICAMP



CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1704-1**, sobre **"Maturação da resposta secretória à glicose pelas ilhotas de Langerhans de ratos recém nascidos induzidas pelo Fator Neurotrófico Ciliar (CNTF)"**, sob a responsabilidade de **Prof. Dr. Antonio Carlos Boschero / Luiz Fernando de Rezende**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **13 de novembro de 2008**.

CERTIFICATE

We certify that the protocol nº **1704-1**, entitled "_____", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **November 13, 2008**.

Campinas, 13 de novembro de 2008.

Prof. Dr. Stephen Hyslop
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