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4-Methlycatechol prevents NGF/p75^{NTR}-mediated apoptosis *via* NGF/TrkA system in pancreatic β cells

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ABSTRACT

In this study, it was aimed to investigate whether 4-methylcatechol (4-MC) could serve as an autocrine antiapoptotic agent by increasing nerve growth factor (NGF) in β cells of hyperglycemic rats. Rats were divided into four groups: the first group was given citrate buffer and saline, the second group was administered 4-MC, the third group received streptozotocin (STZ), and the fourth group was given both 4-MC and STZ. 4-MC (10 μg/kg) was administered by daily intraperitoneal injection for 10 days before the animals were rendered hyperglycemic by administration of STZ (75 mg/kg). With 4-MC pretreatment on hyperglycemic rats the following results were noted: (i) Increase in plasma glucose, β cell apoptosis and caspase-8 activation was prevented. (ii) Reduction of NGF^{+} and tyrosine receptor kinase A (TrkA)$^{+}$ β cell number was blocked. (iii) p75 neurotrophin receptor (p75^{NTR})$^{+}$ β cell number was increased. These data suggest that 4-MC might exert its antiapoptotic actions through NGF/TrkA system which may block NGF/p75^{NTR} activation in pancreatic β cells of hyperglycemic rats.

1. Introduction

Hyperglycemia have been always associated with defects in β cell function in diabetes mellitus (Leahy et al., 1992; Ferrannini, 1998). Diabetes is characterized by loss or dysfunction of β cells. The rate of β cell proliferation, hyperplasia, neogenesis and death combine to determine β cell mass (Bonner-Weir, 2000) and it is influenced by exposure to insulin, glucose and several growth factors (Nielsen et al., 2001). The pancreatic microenvironment can play an important role in β cell death by making these cells more susceptible or resistant to damage. High glucose concentration impairs islet function by disturbing glucose metabolism in the mitochondria of B cells and induces apoptosis (Federici et al., 2001; Laybutt et al., 2001). Apoptosis is a tightly regulated physiological process, triggered by a variety of metabolic or cytokine-dependent stimuli, consisting in a constitutive program that leads to cell death. The initial death signals can activate the apoptotic pathway which involves a cascade of highly regulated hierarchical molecular events (Chandra et al., 2001). These events are mediated by a proteolytic cascade in which upstream activator caspases initiate and amplify the maturation of effector caspases that, in turn, cleave a discrete subset of cellular polypeptides to manifest the apoptotic phenotype (Alnemri, 1997). The activation of proximal caspases, such as caspase 8, in the apoptotic cascade is thought to occur by an autocatalytic mechanism which then leads to the maturation of caspase-3, a key enzyme involved in the terminal apoptotic cascade and cell death (Thornberry and Lazebnik, 1998).

Pancreatic β cells share several biologic properties with neuronal cells including expression of neuronal differentiation factor neuroD/β2 and dependence on nerve growth factor (NGF) *in vitro* (Pierucci et al., 2001). Moreover, multipotent precursors that generate both neural and pancreatic lineages have been isolated from pancreatic ductal cells and islets (Seaberg et al., 2004), suggesting that neurotrophic factors such as NGF might play a role in β cell growth and survival. NGF is an important growth factor for the differentiation and survival of neurons and neural crest-derived cells (Levi-Montalcini, 1987). However, numerous studies reveal that NGF does not only act in the cells of the peripheral and central nervous systems, but also outside the nervous system, especially in the endocrine and immune systems (Aloe et al., 1999; Sariola, 2001). NGF mediates its functions through binding to specific cell surface receptors: p75 neurotrophin receptor (p75^{NTR}), which is the low affinity NGF receptor whose function has not yet been completely elucidated, and tyrosine receptor kinase A (TrkA), which is the high affinity NGF receptor may combine with p75^{NTR} to form a receptor complex with full biological activity (Kaplan and Miller, 2000).

NGF secreted by β cells is an autocrine regulator of survival and preserves insulin biosynthesis and secretion (Navarro-Tableros et al., 2004). These observations may contribute to a better understanding of the physiopathology of diabetes, which insulin and

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NGF levels are diminished. It has been demonstrated that pancreatic β cells express functional receptors for NGF and it exerts some effects on β cells such as increase in insulin secretion and induction of neuron like differentiation (Polak et al., 1993; Miralles et al., 1998; Rosenbaum et al., 1998). Although the role of NGF in pancreatic β cell survival is poorly understood, reports of increased expression of it in these cells following islet injury (Teitelman et al., 1998) raises the possibility that NGF may be involved in β cell survival and development of diabetes.

Previous studies have shown that catecholamines stimulate NGF synthesis in cultured mouse astroglial cells and fibroblasts (Furukawa et al., 1989). It has also been reported that alkylcatechols and their derivates can serve as potential inducer of NGF synthesis *in vivo* and that induced NGF has physiological effects on peripheral neurons. Namely, administration of methylcatechol (MC), one of the alkylcatechols, elicits the elevation of both NGF protein and its mRNA in the peripheral organs, including the heart and submaxillary gland, in adult rats (Kaechi et al., 1993). However, there is no data about the effects of 4-MC on the pancreas.

Based on the above-cited evidences, this study presents, for the first time, the effects of 4-MC on NGF and its receptors in pancreatic β cells of euglycemic or hyperglycemic rats. Our data indicates an important role of NGF and its receptor levels in the regulation of β cell survival, apoptosis, and the physiologic consequence on glucose homeostasis. We determined that elevated NGF and TrkA levels, by 4-MC administration, promoted β cell survival, protected against STZ-induced β cell apoptosis, and improved glycemic control, while depleted NGF and elevated $p75^{NTR}$ levels contributed apoptosis in β cells *in vivo*.

2. Methods

All experiments were carried out in accordance with the guidelines of the Istanbul University local ethic committee of experimental animals. The rats were obtained from Animal Care and Use Committee of Istanbul University Experimental Medical Research and Application Institute DETAE and they were kept at a constant temperature (22 ± 1 °C) with 12 h light and dark cycles and fed with a standard pellet chow.

2.1. Experimental design

All experiments were initiated in adult male Wistar albino rats at 10 weeks of age and 250–300 g body weight after glucose concentration were measured in fasting blood samples to ensure that the rats were not hyperglycemic. The animals were divided into four groups. Group 1: they were taken physiologic saline (0.9% NaCl), every 24 h for 10 days before 0.01 M citrate buffer (pH: 4.5 injection on 11th day and sacrificed 4 h after citrate buffer injection, Group 2: they were treated with 4-MC (10 μg/kg, Sigma, St. Louis, MO) dissolved in physiologic saline (0.9% NaCl), every 24 h for 10 days and sacrificed on 11th day after citrate buffer injection, Group 3: they were injected physiologic saline (0.9% NaCl), every 24 h for 10 days before rendering hyperglycemic by single intraperitoneal injection of STZ (75 mg/kg, Sigma, St. Louis, MO) dissolved in a freshly prepared 0.01 M citrate buffer (pH: 4.5) and sacrificed after 4 h, and Group 4: they were pretreated with 4-MC (10 μg/kg) dissolved in physiologic saline (0.9% NaCl), every 24 h for 10 days before STZ (75 mg/kg) injection on 11th day and sacrificed 4 h after STZ injection. Ten animals were used for each group. The animals were fasted overnight prior to experiment, but they were allowed free access to water. The animals were sacrificed through cervical dislocation.

2.2. Glucose measurement

Fasting blood samples were obtained from tail vein and glucose levels were determined using an automated glucose analyzer (Roche Accu Check Sensor Comfort, Roche Diagnostics, GmbH, Mannheim, Germany).

2.3. Caspase-8 activity measurement

Pancreatic caspase-8 activities were measured by quantifying the cleavage of acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNa) with a colorimetric caspase-8 assay kit (Chemicon-APT171), as instructed by the manufacturer. P-Na level, released by the enzymatic hydrolysis of Ac-IETD-pNa, was calculated from a p-Na standard curve at 405 nm via microtiter plate reader (Biotek). Caspase-8 activity was calculated by subtracting the absorbance measured in the presence of substrate plus inhibitor from the absorbance observed by incubation with substrate alone for 2 h at 37 °C. The activity of the enzyme is expressed as nmol p-NA liberated per mg of protein per hour. The protein concentration of enzyme source was measured using Lowry method (Lowry et al., 1951).

2.4. Double immunofluorescence

The sections of paraffin-embedded, 10% buffered formalin-fixed rat pancreas tissue samples were stained using double immunofluorescence technique. The sections were incubated with one of the following primary antibodies: 1:400 diluted guinea pig anti-insulin (Zymed-180067) at room temperature for 30 min; 1:300 diluted rabbit anti-NGF (Santa cruz-sc549) at 4 °C for overnight; 1:500 diluted rabbit anti-TrkA (Santa cruz-sc118) or rabbit anti-$p75^{NTR}$ (Chemicon-AB1554) at room temperature for overnight diluted in 5% calf serum plus 0.01% Triton X-100 in Tris Buffered Saline (TBS, 50 mM, pH 7.8). TBS was used instead of primary antibody for control sections. Then the secondary antibodies 1:400 diluted texas red (TR)-conjugated goat anti-guinea pig IgG (Santa cruz-scG2996) at room temperature for 30 min and 1:200 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Santa cruz-scG1708) at room temperature for 45 min were applied in 1.5% calf serum plus 0.01% Triton X-100 in TBS, respectively. The immunostained specimens were viewed with a fluorescent microscope (Olympus BX-51 including UMWB2 and UMWG2 filters) by using 40× objective and 10× ocular system. At least 10 islets were counted for per slides from five animals for each group. The immunopositive β cell index was calculated by using following formula:

$$\text{Immun}^{+}\ \beta\ \text{Cell}\ \%: \frac{\sum \text{and Immun}^{+}\ \text{and Insulin}^{+}\ \text{Cell Number}}{\sum \text{Insulin}^{+}\ \text{Cell Number}} \times 100$$

2.5. Immunohistochemistry for detection of apoptotic β cells

Apoptotic β cells were shown by labelling DNA strand breaks with the TdT-mediated dUTP nick end labelling (TUNEL) technique using an apoptosis detection kit (Chemicon, S7101), followed by staining for insulin to detect β cells. Sections were deparaffinised and digested with proteinase K (20 μg/ml for 15 min at 37 °C). Sections were adapted to terminal deoxynucleotidyl transferase (TdT) and dUTP mixture at 37 °C for 1 h. Staining was developed with diaminobenzidine (DAB) as chromogen to obtain a brown reaction in the nucleus. Slides were washed in 0.01% Tween 20/TBS and incubated with mouse anti-insulin (Thermo, MS-1379) diluted 1:400, for 30 min at room temperature. After washing the Histostain Plus Alkaline Phosphatase Broad Spectrum Kit (Zymed,