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Carnosine protects pancreatic beta cells and islets against oxidative stress damage

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ABSTRACT

Islet transplantation is a valid therapeutic option for type 1 diabetes treatment. However, in this procedure one of the major problems is the oxidative stress produced during pancreatic islet isolation. The aim of our study was to evaluate potential protective effects of L-carnosine and its isomer D-carnosine against oxidative stress. We evaluated the carnosine effect on cell growth, cell death, insulin production, and the main markers of oxidative stress in rat and murine stressed beta cell lines as well as in human pancreatic islets. Both isomers clearly inhibited hydrogen peroxide induced cytotoxicity, with a decrease in intracellular reactive oxygen and nitrogen species, prevented hydrogen peroxide induced apoptosis/necrosis, nitrite production, and reduced glucose-induced insulin secretion. In addition, NF-κB expression/translocation and nitrated protein induced in stressed cells was significantly reduced. Furthermore, both isomers improved survival and function, and decreased reactive oxygen and nitrogen species, and nitrite and nitrotyrosine production in human islets cultured for 1, 3, and 7 days. These results seem to indicate that both L and D-carnosine have a significant cytoprotective effect by reducing oxidative stress in beta cell lines and human islets, suggesting their potential use to improve islet survival during the islet transplantation procedure.

1. Introduction

Diabetes mellitus is a worldwide health problem, with significant economic and social costs. Oxidative stress is a common feature of both type 1 (T1) and type 2 (T2) diabetes mellitus (DM), and biomarkers of oxidative stress are consistently elevated in the pancreas and other tissues of diabetic patients (Robertson, 2006).

In T1DM, Langerhan's insulin-producing pancreatic beta cells are destroyed by the immune system (Grieco et al., 2012), and islet transplantation (ITx) of isolated pancreatic islets has become a suitable intervention strategy for treatment (Alejandro et al., 2008; Balamurugan et al., 2014; Barton et al., 2012; Hering, 2005; Hering et al., 2016; Shapiro et al., 2005). The process of pancreatic islet isolation is a combination of sequential mechanical and enzymatic dissociation, followed by gradient separation (Ricordi et al., 1989), and in recent years, various improvements in the isolation of islets have been introduced to enhance the success rates of pancreatic ITx (Linetsky et al., 1997; Rheinheimer et al., 2015; Ricordi et al., 2016). Indeed, during islet isolation procedures, islets are exposed to different insults in which oxidative stress plays an important role and leads to islet death and cell loss, thus contributing to insufficient engraftment and re-

Abbreviations: CI, CELL index; D-CA, D-carnosine; L-CA, L-carnosine; NAC, N-acetyl-L-cysteine; DCF-DA, 2′,7-dichlorofluorescein-diacetate; GSIS, glucose stimulated insulin secretion; H_2O_2, hydrogen peroxide; ITx, islet transplantation; NO_2, nitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

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establishment of metabolic function. Several *in vitro* and *in vivo* studies have shown that mechanical and enzymatic insults during islet isolation and transplantation, as well as high glucose exposure, lead to oxidative stress, influencing beta cell survival and function (Armann et al., 2007; Harmon et al., 2005; Paraskevas et al., 2000; Stiegler et al., 2010; Tanaka et al., 1999). This problem is generated by the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity (Wang et al., 2013; Taibi et al., 2010; Slimen et al., 2014).

Pancreatic beta cells are highly vulnerable to oxidative stress due to their lack of a robust antioxidant system. Indeed, a low expression and activity of the hydrogen peroxide (H_2O_2)-inactivating enzymes in the insulin-producing beta cells compared with other tissues has been observed (Lenzen, 2008; Tiedge et al., 1997). The high susceptibility of pancreatic beta cells to free-radical-induced oxidative damage prompted us to test the effect of carnosine on insulin-producing cells. Carnosine is a dipeptide (beta-alanine and histidine) with an antioxidant protective effect, and naturally expressed in several organs, such as muscles (Abe, 2000) and the brain (Bonfanti et al., 1999). It has been suggested that L-carnosine (beta-alanyl-L-histidine) has antioxidant and free radical scavenging functions, which may partially explain its apparent homeostatic function (Aruoma et al., 1989). However, the biological roles of carnosine and similar dipeptides are still unknown (Bellia et al., 2012), though their possible role as proton buffering agents in muscle tissues (Abe, 2000) and as an effector on metabolism has been suggested (Renner et al., 2010). Moreover, carnosine has shown the ability to reduce nitrosative stress (Calabrese et al., 2005), acting as a carbonyl-scavenger (Aldini et al., 2011), and promoting antioxidant activity (Bellia et al., 2008). Carnosine effects have been shown in pathological conditions like diabetes (Janssen et al., 2005; Sauerhofer et al., 2007), and several studies have revealed an association between carnosinase activity and diabetic nephropathy (Bellia et al., 2014). In these studies, an overexpression of carnosinase, leading to an increase in carnosine degradation, and results in a lower renal protective effect of carnosine (Janssen et al., 2005).

Beta cells are exposed to oxidative stress during different steps in ITx, such as pancreas preservation and islet isolation, during and after transplantation. Moreover, islets are subjected to a significantly prolonged ischemic period compared with other transplanted organs. Unlike other organs, transplanted islet ischemia does not end immediately after transplantation. Indeed, revascularization has been shown to take about 10 days post-ITx in a mouse model (Vajkoczy et al., 1995). Given the role of ischemia reperfusion and prolonged ischemia in ROS production and tissue damage, this represents another critical step during ITx. In this scenario, carnosine and its derivatives are antioxidant molecules that could improve the outcome of islet survival by decreasing oxidative stress and attenuating inflammatory mediator response (Menini et al., 2012).

To assess the protective effect of carnosine in pancreatic beta cells, we tested both L-carnosine isomer (L-CA) and D-carnosine isomer (D-CA) on stressed insulin-producing cells, using two different cell lines, INS-1E and MIN6 (rat and mouse insulinoma, respectively). In addition, the potential protective effects of D- and L-carnosine were evaluated on human pancreatic islets cultured for 1, 3, and 7 days. Moreover, these data were compared with those obtained by treatment with *N*-acetyl-L-cysteine (NAC), a potent antioxidant. The aim of our study was to establish whether L-CA and D-CA (alone or in combination) can improve pancreatic beta cell viability and function through protection against oxidative stress in two different cell lines and in human pancreatic islets.

2. Materials and methods

2.1. Cell culture and treatments

Rat INS-1E beta cell lines were maintained in RPMI1640 medium with 11 mM glucose (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 10 mmol/L HEPES, 1 mM sodium pyruvate, 2 mmol/L L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, USA) at 37 °C and 5% CO_2. Mouse MIN6 beta cell lines were grown in DMEM with 25 mM glucose (Invitrogen, USA) containing 15% FBS (FBS, Hyclone, USA), 2 mmol/L l-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, USA) at 37 °C and 5% CO_2.

D-carnosine (provided by Flamma), L-carnosine and *N*-acetyl-L-cysteine (Sigma, St. Louis, MO, USA) lyophilized powder was dissolved in sterile water and added to the cell plates overnight at a final concentration of 10 mM before inducing oxidative stress with H_2O_2 treatment at different incubation times and final concentrations.

2.2. Cell growth assay (xCELLigence)

Cell growth was measured in real time using xCELLigence technology (ACEA, San Diego, CA), and according to the vendor's instructions. The cells were pre-incubated with or without (controls) D- and/or L-carnosine (10 mM) and NAC (10 mM) before treatment with 200 μM hydrogen peroxide (H_2O_2) in the log growth phase (after 48 h for INS-1E cells, and after 24 h for MIN6). The time point 0 h was established when cells were exposed to oxidative stress. The cells were treated with H_2O_2 during the entire experiment. The impedance was measured every 15 min and expressed as cell index (CI), defined as $(R_n-R_b)/15$, where R_n is the impedance at a given time point, and R_b is the background impedance. Each culture condition was carried out in quadruplicate. The normalized CI was determined by the CI at a given time point divided by the CI at the normalization time point. Slopes of growth curves were determined between the time points at days 1 and 2.

2.3. Cell morphology

Cell morphology was assessed with an inverted phase contrast light microscope (Olympus BH-2, Olympus Optical Co., Tokyo, Japan) at × 4 magnification. The cells, at 50% confluence, were pre-incubated with or without D- and/or L-carnosine (10 mM), and then incubated with or without (controls) 200 μM H_2O_2 for 24 h. Digital images were obtained with a digital camera system (Olympus optical imaging, LC-20, Tokyo, Japan).

2.4. Intracellular ROS/RNS levels assay

Intracellular ROS/RNS levels were measured by the dichlorofluorescein (2′,7′-DCF-DA) assay. 2′,7′-DCF-DA (Sigma, St. Louis, MO, USA) reacts quantitatively with intracellular radicals to generate DCF, a fluorescent product that is retained within the cells (Murrant and Reid, 2001). The cells were pre-incubated with or without D- and/or L-carnosine (10 mM) and NAC (10 mM), then incubated with or without 200 μM H_2O_2 for 60 min. The cells were then washed twice with PBS and incubated with 10 μM DCF-DA for 40 min at 37 °C, and then washed twice with PBS. Fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a FACS ARIA II instrument, and analyzed with Diva 6.1.2 software (Becton Dickinson Biosciences). The experiments were carried out in triplicate.

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