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Microbial phenolic metabolites improve glucose-stimulated insulin secretion and protect pancreatic beta cells against tert-butyl hydroperoxide-induced toxicity via ERKs and PKC pathways

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ABSTRACT

Oxidative stress is accepted as one of the causes of beta cell failure in type 2 diabetes. Therefore, identification of natural antioxidant agents that preserve beta cell mass and function is considered an interesting strategy to prevent or treat diabetes. Recent evidences indicated that colonic metabolites derived from flavonoids could possess beneficial effects on various tissues. The aim of this work was to establish the potential anti-diabetic properties of the microbial-derived flavonoid metabolites 3,4-dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA) and 3-hydroxyphenylpropionic acid (HPPA). To this end, we tested their ability to influence beta cell function and to protect against tert-butyl hydroperoxide-induced beta cell toxicity. DHPAA and HPPA were able to potentiate glucose-stimulated insulin secretion (GSIS) in a beta cell line INS-1E and in rat pancreatic islets. Moreover, pre-treatment of cells with both compounds protected against beta cell dysfunction and death induced by the pro-oxidant. Finally, experiments with pharmacological inhibitors indicate that these effects were mediated by the activation of protein kinase C and the extracellular regulated kinases pathways. Altogether, these findings strongly suggest that the microbial-derived flavonoid metabolites DHPAA and HPPA may have anti-diabetic potential by promoting survival and function of pancreatic beta cells.

1. Introduction

Type 2 diabetes (T2D) is a complex metabolic disorder considered a major health problem because of its high frequency and its associated complications resulting in blindness, kidney failure, heart disease, stroke and amputations (Chaturvedi, 2007). There are currently 366 million people worldwide with diabetes and the incidence of the disease is projected to be more than double by 2030 (Whiting et al., 2011) T2D results from a combination of genetic and acquired factors that impair beta cell function and tissue insulin sensitivity but there is growing evidence that beta cell dysfunction is crucial for the development and progression of the disease (Marchetti et al., 2008). Indeed, the inability of beta cells to secrete adequate amounts of insulin results in chronic hyperglycemia and excessive oxidative stress which has been largely implicated in the pathogenesis of pancreatic beta cell failure (Bensellam et al., 2012). This scenario leads to a vicious circle that contributes to the decline of the functional beta cell mass and the consequent progression of T2D (Poitout and Robertson, 2008) Therefore, treatments aimed at enhancing beta cell mass and function are considered key to prevent or treat diabetes and retard the onset of

Abbreviations: AKT/PKB, protein kinase B; DCF, dichorofluorescein; DHBA, 2,3-dihydroxybenzoic acid; DHPAA, 3,4-dihydroxyphenylacetic acid; ERK, extra cellular regulated kinase; GSIS, glucose-stimulated insulin secretion; HPPA, 3-hydroxyphenylpropionic acid; KRB, Krebs–Ringer bicarbonate buffer; PKA, protein kinase A; PKC, protein kinase C; T2D, type 2 diabetes; *t*-BOOH, tert-butyl hydroperoxide; PI3K, phosphatidylinositol-3-kinase.

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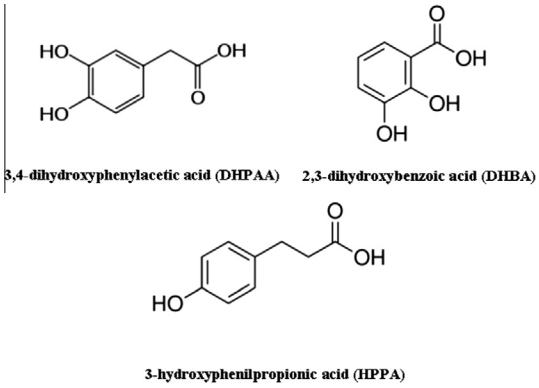

Fig. 1. Chemical structure of 3,4-dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA) and 3-hydroxyphenylpropionic acid (HPPA).

diabetic complications (Robertson, 2010). Furthermore, due to the central role of oxidative stress in the progression and development of T2D, the interest for the identification of natural antioxidant compounds with these characteristics has started to grow rapidly.

Flavonoids are a group of polyphenols abundant in fruits and vegetables with demonstrated antioxidant and anti-inflammatory properties (Del Rio et al., 2013). Interestingly, health effects of flavonoids in humans are dependent of their bioavailability. While monomers are readily absorbed in the small intestine, oligomers and polymers reach intact the colon where they are metabolized by the intestinal microbiota into various phenolic acid derivatives of low molecular weight (Van't Slot et al., 2010; Del Rio et al., 2013). The resultant microbial metabolites are further absorbed into the blood stream and circulate in the body prior to excretion in urine, representing a very large percentage of the amount ingested. Consequently, attention is now focused on the study of the bioactivity of microbial-derived metabolites as compounds responsible for the health effects of flavonoids. Mono- and dihydroxylated phenylpropionic and phenylacetic acids have been found as main microbial phenolic acids derived from flavanol intake, mainly cocoa and cocoa derived products but also grape seed, tea and wine (Monagas et al., 2010). Current evidences indicate that these flavonoid metabolites produced by colonic microbiota could possess biological properties and therefore potential health beneficial effects (Monagas et al., 2010). In particular, colonic metabolites have demonstrated anti-oxidant (Qiu et al., 2013), anti-trombogenic (Rechner and Kroner, 2005) and anti-inflammatory activities (Monagas et al., 2009; Larrosa et al., 2009) on various tissues. More recently, it has been shown that some polyphenolic metabolites were also able to exert insulin-like activities (Scazzocchio et al., 2011) and to counteract two key features of diabetic complications, i.e. protein glycation and neurodegeneration (Verzelloni et al., 2011). Altogether, these results support the hypothesis that not only food polyphenols but also their microbial metabolites must be taken into account when assessing the impact of polyphenols on health (Cardona et al., 2013). However, up to date, whether these phenolic acids have a direct effect on pancreatic beta cells is unclear.

Te aim of the present study was to establish the potential antidiabetic properties of the microbial-derived flavonoid metabolites 3,4-dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA) and 3-hydroxyphenylpropionic acid (HPPA) (Fig. 1). To this end, using a pancreatic beta cell line (INS-1E cells) and isolated rat islets we investigated the effects of DHPAA, DHBA and HPPA on insulin secretion and the mechanisms underlying their action. Besides, their potential ability to protect against oxidative stress-induced beta cell toxicity was also evaluated.

2. Material and methods

2.1. Materials and chemical

3,4-Dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA), 3-hydroxyphenilpropionic acid (HPPA), gentamicin, penicillin G, streptomycin, bovine serum albumin (fraction V), tert-butyl hydroperoxide (*t*-BOOH) and PD98059 were purchased from Sigma Chemical (Madrid, Spain). Collagenase P was obtained from Roche (Barcelona, Spain). RO320432 and H89 were purchased from Calbiochem (Millipore, Madrid, Spain). The fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Anti-extra cellular regulated kinases (ERKs), antiphospho-ERKs (p-ERKs) and anti-β-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-protein kinase C alfa (PKCα) and antiphospho-PKCα (p-PKCα) were purchased from Santa Cruz (sc-208 and sc-12356, respectively, Quimigen, Madrid, Spain). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (BioRad Laboratories S.A., Madrid, Spain). Cell culture medium and foetal bovine serum (FBS) were from Lonza (Madrid, Spain).

2.2. Cell culture

Rat INS-1E cells (a gift from Dr. Mario Vallejo, Instituto de Investigaciones Biomédicas Alberto Sols, CSIC, Madrid, Spain) were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in RPMI-1640 medium with 11 mM glucose, supplemented with 10% FBS, 1% Hepes, 1 mM sodium pyruvate, 50 μM betamercaptoethanol and 1% of the following antibiotics: gentamicin, penicillin and streptomycin.

2.3. Rat islet isolation

Pancreatic islets were isolated from 70-day-old male Wistar rats by a collagenase digestion procedure (Martín et al., 1997). Briefly, islets were isolated from the pancreases of two or three rats and subsequently separated from the remaining exocrine tissue by hand-picking under a dissecting microscope. The islets were immediately used for experiments. All animal care and experimental procedures were performed according to the Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas, CSIC).

2.4. DHPAA, DHBA and HPPA treatments

The different concentrations of DHPAA, DHBA and HPPA (1, 5 and 10 μM) were diluted in RPMI-1640 culture medium and added to the cell plates for 20 h. In the experiments with the pharmacological inhibitors, cells were preincubated with 5 μM PD98059 (PD), 10 μM H89 or 5 μM RO320432 (RO) for 1 h prior to DHPAA, DHBA and HPPA treatment for 20 h. To evaluate the protective effect of microbial phenolic metabolites against an oxidative stress, after DHPAA, DHBA and HPPA treatment the medium was discarded and fresh medium containing 50 μM of *t*-BOOH was added at different times.

2.5. Evaluation of cell viability and ROS production

Cell viability was determined by using the crystal violet assay. INS-1E cells were seeded at low density (2×10^5 cells per well) in 24-well plates. After the different treatments, cells were incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1% sodium dodecyl sulfate (SDS) added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

Cellular ROS were quantified by the DCFH assay using a microplate reader. For the assay, cells were plated in 24-well multiwells and incubated with the different treatments. After that, 10 μM DCFH was added to the wells for 30 min at 37 °C. After being oxidized by intracellular oxidants, DCFH will become dichorofluorescein (DCF) and emit fluorescence. ROS generation was evaluated in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

2.6. Insulin secretion and content

In order to evaluate glucose-stimulated insulin secretion (GSIS), after the different treatments, INS-1E cells or islets were washed and placed in Krebs–Ringer bicarbonate buffer (KRB: 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$ $6H_2O$, 1 mM $CaCl_2$ $2H_2O$) supplemented with 5 mg/mL BSA for a quiescent period of two hours. Next, cells and islets were incubated for 90 min in KRB containing 4 or 10 mM glucose and the different concentrations of DHPAA, DHBA and HPPA. For the oxidative stress experiments, the 90 min of incubation was performed out in the presence of 50 μM of *t*-BOOH. Insulin secreted in the medium was evaluated by an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden).