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Saxagliptin prevents vascular remodeling and oxidative stress in db/db mice. Role of endothelial nitric oxide synthase uncoupling and cyclooxygenase

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ABSTRACT

To explore the hypothesis that DPP-IV are involved in the diabetes-induced vascular damage, we assessed the vascular effects of chronic administration of saxagliptin (Saxa) or metformin (Met) in db/db mice, a model of type 2 diabetes, evaluating vascular structure and endothelial function in mesenteric small arteries. The increases in media/lumen and media cross sectional area were prevented by Saxa. In db/db, the blunted response to acetylcholine was only marginally affected by L-NAME (NO-synthase inhibitor), improved by SC-560 (cyclooxygenase-1 inhibitor) or SQ-29548 (thromboxane receptor antagonist), and totally restored by Apocynin (NAD(P)H-oxidase inhibitor). DFU (cyclooxygenase-2 inhibitor) had no effect. Saxa improved acetylcholine-induced relaxation, which returned partially sensitive to the inhibition of L-NAME. Dihydroethidium staining revealed an increased intravascular superoxide production in db/db, attenuated by L-NAME and Saxa, and abrogated by apocynin. The dimer/monomer ratio of endothelial NOS was decreased in db/db mice and restored by Saxa. Cyclooxygenase-1 and thromboxane-A_2 receptor expression, higher in db/db, was down-regulated by Saxa. Met treatment did not modify any of the abnormal vascular responses.
Saxa reverses vascular hypertrophic remodeling and ameliorates NO availability in small arteries from db/db mice through the abrogation of NAD(P)H oxidase-driven eNOS uncoupling and by reducing the action of cyclooxygenase-1-derived vasoconstrictors downregulating the expression of thromboxane-prostanoid receptors.

1. Introduction

Type 2 diabetes is characterized by vascular structural and functional alterations (vascular remodeling and endothelial dysfunction, respectively) both in large and small-resistance arteries [1,2]. In particular, at the level of peripheral microcirculation, increased media to lumen ratio (M/L), a hallmark of vascular remodeling, mainly results from a thicker media encroaching on the lumen (hypertrophic remodeling) [3]. Endothelial dysfunction is caused by a reduced nitric oxide (NO) availability secondary to increased production of reactive oxygen species (ROS). NAD(P)H oxidase is a major ROS source in this clinical condition [4]. Also cyclooxygenases (COX) are involved in the diabetes-induced vascular dysfunction.

Briefly, COX triggers the generation of bioactive prostanoids. In physiological conditions, prostacyclin is the major prostanoid mediating several vascular protective effects, while its opponent is the vasoconstricting thromboxane (TX)A2, specifically acting on thromboxane-prostanoid (TP) receptors. Two distinct COX isoenzymes, COX-1 and COX-2, have been described [5]. Previous reports in animal models of type 2 diabetes documented an enhanced release of COX-2-derived constricting prostanoids [6].

Given the crucial importance played by both vascular functional and structural changes in the pathophysiology of the aggressive atherosclerotic process that often coexists with type 2 diabetes and their role as independent prognostic markers of cardiovascular events [7,8], it is likely that they represent important therapeutic targets in the prevention or delay of atherothrombosis in type 2 diabetes.

A novel therapeutic approach to type 2 diabetes is represented by gliptins, a class of drugs able to antagonize the enzyme DPP-IV, thus prolonging the physiological actions of the incretin hormones (GLP-1, GIP), with a clinically relevant reduction in plasma glucose levels [9]. DPP-IV, however, are ubiquitous enzymes, highly expressed in the membrane of a variety of cells [10,11]; as a consequence, gliptins may beneficially affect chronic low grade inflammation and oxidative stress in type 2 diabetes [12,13]. Among DPP-IV inhibitors, saxagliptin (Saxa) has shown anti-inflammatory properties, enhancing NO release and reducing circulating levels of some cytokines in obese and in hypertensive rats [14,15]. However, a recent intriguing report raised doubts on the

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real protection exerted by these compounds on the vasculature, describing an unexpected attenuation of endothelial function, as measured by flow-mediated dilation, occurring in type 2 diabetic patients treated with sitagliptin during a specifically-designed cross-over trial [16]. In such scenario, a better definition of the direct vascular actions of DPP-IV inhibitors in type 2 diabetes appears necessary, particularly when considering the increased cardiovascular risk associated to type 2 diabetes, together with the widespread use of this class of anti-hyperglycemic drugs. Therefore, we utilized a mouse model of type 2 diabetes to investigate the effects of chronic Saxa administration on vascular remodeling and endothelial dysfunction, focusing on NO availability and NAD(P)H oxidase-derived ROS generation. The activation of COX isoforms, as possible pathways exploited by Saxa in this vascular district, was also assessed.

2. Methods

2.1. Animals

Sixteen week-old male adult mice C57BLKS/J (WT) and BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J (db/db) were housed in standard cages with free access to water and standard chow, under controlled ambient illumination on a 12 h light/dark cycle. An equal number of mice (n = 25) in each group received Saxa (0.1 mg/kg/die) or vehicle in drinking water for 8 weeks. Another db/db mice subset (n = 8) was treated for the same period with metformin (Met, 300 mg/kg/die, same route of administration) as internal control of the glucose lowering effect. Plasma glucose concentration, systolic blood pressure (by the tail-cuff method) and body weight were recorded every week. Experimental protocol was approved by the Ethic Committee for Animal Research of the University of Pisa (approval reference number 12498, October 3, 2012). At the end of the treatments, animals were sacrificed; blood was collected and tissues were explanted. All the procedures were performed according to the rules of the EU-Convention on the protection of animals used for scientific purpose.

2.2. Chemicals and reagents

Acetylcholine, *sodium nitroprusside*, *norepinephrine*, *KR-62436*, *L-NAME*: from Sigma Chemicals, St. Louis, USA; *Apocynin*: from Fluka, Buchs, Switzerland.

Primary antibodies: COX-1, COX-2 and TP from Cayman Chemical Company, Ann Arbor, USA;

eNOS, iNOS from abcam, Cambridge, UK; β-actin from Santa Cruz Biotechnology, Santa Cruz, USA; *Secondary antibodies*: from Chemicon International, Temecula, USA.

miRCURY™ Isolation Kit: from Exiqon, Vedbaeck, Denmark; *cDNA Reverse Transcription Kit*: from Life Technologies, Carlsbad, USA; *Any kD Mini-Protean TGX gels*: from Bio-Rad, Segrate, Italy; *PVDF membrane*: from Millipore, Billerica, USA; *Enzymatic chemiluminescence kit*: from Immobilon Western, Millipore, USA.

2.3. Preparation of small arteries, vascular morphology and endothelium-dependent relaxation

Second-order branches of the mesenteric arterial tree were dissected and placed in cold physiological salt solution (PSS) which contained (mmol/l): NaCl 120, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5, EDTA 0.026, and glucose 5.5, with pH = 7.4, mounted on 2-glass microcannulae in a pressurized myograph chamber and equilibrated as previously described [17,18]. Media and lumen dimensions were measured with a constant intraluminal pressure of 45 mm Hg, set with a servo-controlled pump. Media cross-sectional area (CSA) was estimated as previously described [19]; the growth index was calculated as (CSA_d − CSA_c)/CSA_c, where CSA_c and CSA_d were media CSA of vessels from WT and db/db animals, respectively. An increased media CSA indicated the hypertrophic nature of the vascular remodeling

Endothelium-dependent relaxation was assessed by measuring dilatory responses to cumulative concentrations of acetylcholine (Ach, 1 nmol/l to 100 μmol/l). Endothelium-independent relaxation was assessed by sodium nitroprusside (0.01–100 μmol/l). Vessels were pre-contracted with norepinephrine (10 μmol/l), whose concentration was chosen according to preliminary dose-titration experiments to establish the threshold concentration able to elicit similar contractions among the experimental groups (data not shown). Only vessels that responded with >70% vasoconstriction to extraluminal application of 0.9% KCl + 10 μM norepinephrine were used.

To confirm that the Saxa-induced responses would have been specifically mediated by an inhibition of the DPP-IV enzyme, in an adjunctive group of six db/db mice, some experiments were repeated after 30 min pre-incubation with KR-62436 (6-[2-[2-(5-cyano-4,5-dihydropyrazol-1-yl)-2-oxoethylamino]ethylamino] nicotinonitrile), a competitive chemical inhibitor of DPP-IV (100 μmol/l). This concentration was selected on the basis of preliminary concentration-response experiments (data not shown) as well as a previous report evidencing a suppression of plasma DPP-IV activity [20].

2.4. Influence of NO availability and NAD(P)H oxidase on endothelium-dependent relaxation

To evaluate NO availability, concentration-response curves to Ach were constructed before and after 30 min pre-incubation with the NO synthase (NOS) inhibitor N$^{\omega}$-nitro-L-arginine methylester (L-NAME, 100 μmol/l). To assess the influence of NAD(P)H oxidase on endothelial function, Ach was tested after 30 min incubation with the NAD(P)H oxidase inhibitor apocynin (10 μmol/l) and diphenylene iodinium (DPI, 10 mmol/l). Finally, to assess whether NAD(P)H oxidase was involved in decreasing NO availability, Ach was tested during simultaneous incubation with L-NAME plus apocynin. Concentration of apocynin and DPI were selected according to previous studies reporting the concentrations able to inhibit the respective enzymatic activities [21].

2.5. Involvement of COX-1, COX-2 activity and TP receptors on endothelium-dependent relaxation

The participation of COX-1 and COX-2 isoenzymes on endothelial function was assessed by construction of concentration-response curves to Ach after 30 min pre-incubation with SC-560 (1 μmol/l; selective COX-1 inhibitor) or DFU (1 μmol/l; selective COX-2 inhibitor). To ascertain the contribution of TP receptors, Ach was repeated after 30-min incubation with SQ-29548 (1 μmol/l; TP receptor antagonist).

2.6. Detection of vascular superoxide anion generation

The *in situ* production of superoxide anion was measured by the fluorescent dye dihydroethidium (DHE), as previously described [21]. Each segment was analyzed simultaneously after incubation with SC-560 (100 μmol/l), L-NAME (100 mmol/l), apocynin (100 μmol/l), ascorbic acid (100 μmol/l) or Krebs solution.

2.7. mRNA and protein expression

1 μg of total RNA, extracted from mesenteric arteries using the miRCURY™ Isolation Kit, was retro-transcribed with High Capacity cDNA Reverse Transcription Kit in an MJ mini thermocycler (Biorad, Hercules, USA). Real-time PCR was performed in triplicate on an Eco-Real Time instrument (Illumina, San Diego, USA) following a standard protocol with specific TaqMan Gene Expression Assays. Primers were: eNOS (Mm00435217_m1), iNOS (Mm00440502_m1), COX-1 (Mm00477214_m1), COX-2 (Mm00478374_m1), β-ACT