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Research Paper

Hydrogen peroxide derived from NADPH oxidase 4- and 2 contributes to the endothelium-dependent vasodilatation of intrarenal arteries

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ABSTRACT

The role of NADPH oxidase (Nox)-derived reactive oxygen species in kidney vascular function has extensively been investigated in the harmful context of oxidative stress in diabetes and obesity-associated kidney disease. Since hydrogen peroxide (H_2O_2) has recently been involved in the non-nitric oxide (NO) non-prostanoid relaxations of intrarenal arteries, the present study was sought to investigate whether NADPH oxidases may be functional sources of vasodilator H_2O_2 in the kidney and to assess their role in the endothelium-dependent relaxations of human and rat intrarenal arteries. Renal interlobar arteries isolated from the kidney of renal tumor patients who underwent nephrectomy, and from the kidney of Wistar rats, were mounted in microvascular myographs to assess function. Superoxide ($O_2^{\cdot-}$) and H_2O_2 production was measured by chemiluminescence and Amplex Red fluorescence, and Nox2 and Nox4 enzymes were detected by Western blotting and by double immunolabeling along with eNOS. Nox2 and Nox4 proteins were expressed in the endothelium of renal arterioles and glomeruli co-localized with eNOS, levels of expression of both enzymes being higher in the cortex than in isolated arteries. Pharmacological inhibition of Nox with apocynin and of CYP 2C epoxygenases with sulfaphenazol, but not of the NO synthase (NOS), reduced renal NADPH-stimulated $O_2^{\cdot-}$ and H_2O_2 production. Under conditions of cyclooxygenase and NOS blockade, acetylcholine induced endothelium-dependent relaxations that were blunted by the non-selective Nox inhibitor apocynin and by the Nox2 or the Nox1/4 inhibitors gp91ds-tat and GKT136901, respectively. Acetylcholine stimulated H_2O_2 production that was reduced by gp91ds-tat and by GKT136901. These results suggest the specific involvement of Nox4 and Nox2 subunits as physiologically relevant endothelial sources of H_2O_2 generation that contribute to the endothelium-dependent vasodilatation of renal arteries and therefore have a protective role in kidney vasculature.

1. Introduction

Oxidative stress and the associated endothelial dysfunction are key pathogenic factors underlying the vascular complications of metabolic disease including diabetic nephropathy [1,2]. However, reactive oxygen species (ROS) can act as both physiological and pathophysiological signaling molecules in the vascular wall. ROS like H_2O_2 have been involved in the endothelium-derived hyperpolarization (EDH) and vasodilation of arterioles from vascular beds such as the coronary circulation, wherein H_2O_2 couples coronary blood flow to myocardium metabolism [3–5]. H_2O_2 released from the endothelium by flow or agonists causes arterial relaxation through activation of K^+ channels and hyperpolarization of vascular smooth muscle (VSM) and by promoting Ca^{2+} release from endothelial cell stores [4,6,7]. Sources of

Abbreviations: ACh, acetylcholine; COX, cyclooxygenase; CYP, cytochrome P450; EC, endothelial cell; EDH, endothelium-derived-hyperpolarization; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; H_2O_2, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; Nox, NADPH oxidase enzymes; $O_2^{\cdot-}$, superoxide; Phe, phenylephrine; PSS, physiological saline solution; ROS, reactive oxygen species; SOD, superoxide dismutase; VSM, vascular smooth muscle

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vascular ROS include endothelial NOS (eNOS) [3], the mitochondria [8], the endothelial NADPH oxidases (Nox) [9], the cyclooxygenases (COX) [10] and the cytochrome P450 (CYP) epoxygenases [11].

The role of ROS in kidney vascular function has extensively been investigated in the context of harmful NADPH oxidase- and mitochondria-derived ROS generation and oxidative stress in diabetes, hypertension and obesity-associated kidney disease [1,12–16], but little is known about the involvement of species such as H_2O_2 in the endothelial-dependent renal vasodilatation. NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular O_2 through the Nox catalytic subunit to generate ROS and is the predominant source of renal superoxide ($O_2^{\cdot-}$). In the kidney, NADPH oxidase enzymes (Nox) are widely distributed in renal vessels, glomeruli and nephron segments, Nox4 and also Nox2 being the predominant forms [17–19]. NADPH oxidase has been found to be a major source of ROS generation, oxidative stress and renal injury mostly under pathological conditions such as diabetic nephropathy and chronic kidney disease [15,16,19]. However, Nox isoforms have also been involved in kidney physiological processes such as regulation of glucose production and handling in the proximal tubule, tubule-glomerular feed-back in macula densa, regulation of renal tubule electrolyte transport and regulation of afferent arteriole responsiveness [18,20,21].

We have recently demonstrated that endothelial-derived H_2O_2 is involved in the non-nitric oxide (NO) non-prostanoid EDH relaxations of rat intrarenal arteries through various mechanisms including stimulation of endothelial K^+ channels and hyperpolarization, and Ca^{2+}-dependent and Ca^{2+} sensitization mechanisms in VSM [22]. Moreover, in our study CYP epoxygenases were identified as relevant enzymatic sources of H_2O_2 generation in the renal endothelium under physiological conditions. Nox2 and Nox4 mRNA have been identified in intrarenal arteries of the human kidney although their functional significance and the role of Nox subunits in renal hemodynamics remain unclear [18]. Furthermore, the predominant Nox isoform in the kidney, Nox4, can have potential beneficial effects on vasodilator function [23]. Therefore, the present study was designed to investigate whether NADPH oxidase may be a relevant functional source of vasodilator H_2O_2 in the renal endothelium and assess its role in the endothelium-dependent vasodilation of human and rat intrarenal arteries.

2. Materials and methods

2.1. Animal model

In the present study, 12–14 weeks old male Wistar rats were housed at the Pharmacy School animal care facility and maintained on standard chow and water ad libitum. All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Institutional Animal Care and Use Committee of Madrid Complutense University. Animals were killed and the kidneys quickly removed and placed in cold (4 °C) physiological saline solution (PSS) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.17, $MgSO_4$ 1.18, $CaCl_2$ 1.5, EDTA 0.027 and glucose 11, continuously gassed with a mixture of 5% CO_2/95% O_2 to maintain pH at 7.4.

2.2. Patients

Human renal tissues were obtained from renal tumour patients who underwent nephrectomy. The investigation with human tissue conformed to the principles outlined in the Declaration of Helsinki. Permission was obtained from the Ethics Committee of the University Hospital Puerta de Hierro-Majadahonda, Spain (Reg. no 5.16) and patients gave their informed consent. We investigated tissues from 9 patients (2 female, 7 males) with a mean ± SEM age of 65 ± 3 years. Out of these patients, 1 was diagnosed with diabetes mellitus and 1 with hidronephrosis and kidneys were discarded from the study. Plasma creatinine levels were 1.02 ± 0.20 mg/ml (n = 7).

2.3. Dissection and mounting of microvessels

Tissue samples from tumour-free parts of the kidney were obtained after kidney resection in the Pathology laboratory, placed in ice-cold physiological salt solution (PSS) and taken to the laboratory for dissection. Microdissection under the microscope of tumor-free kidney samples from 7 patients containing renal cortex and medulla, yielded 20 distal interlobar and arcuate arteries. Small samples of both renal arteries and cortex were also dissected out for ROS measurements.

Renal interlobar arteries, second- or third order branches of the renal artery from Wistar rats, were carefully dissected by removing the medullary conective tissue and mounted in parallel in double microvascular myographs (Danish Myotechnology, Denmark) by inserting two 40 μm tungsten wires into the vessel lumen. After mounting the arteries were equilibrated for 30 min in PSS maintained at 37 °C. The relationship between passive wall tension and internal circumference was determined for each individual artery and from this, the internal circumference, L_{100} corresponding to a transmural pressure of 100 mm Hg for a relaxed vessel *in situ* was calculated. The arteries were set to an internal diameter l_1 equal to 0.9 times l_{100} ($L_1 = 0.9 \times L_{100}$), since force development in intrarenal arteries is close to maximal at this internal lumen diameter [10].

2.4. Experimental procedures for the functional experiments

At the beginning of each experiment, arteries were challenged twice with 120 mM K^+ solution (KPSS) in order to test vessel viability. Endothelium-dependent vasodilatation of renal arteries was assessed by the relaxant effects of acetylcholine (ACh) upon addition of cumulative concentrations of this agent on arteries precontracted with phenylephrine (Phe) and previously incubated with L-NOARG (100 μM) and indomethacin (0.3 μM) to block NOS and COX enzymes, respectively. The responses to exogenous ACh were further obtained in the absence and presence of the SOD scavenger tempol (30 μM), the specific mitochondrial superoxide scavenger MitoTEMPO (1 μM), the non-selective Nox inhibitors apocynin (30 μM) and plumbagin (1 μM), the dual Nox1/4 inhibitor GKT137831 (0.1 μM) and the Nox2 inhibitor Nox2ds-tat (1 μM) [24]. The drugs were added to the myograph chamber 30 min before a second concentration-response curve was performed, and the Phe concentration was adjusted to match the contraction during the first control curve assessment.

2.5. Measurement of superoxide production by chemiluminescence

Changes in basal and NADPH-stimulated levels of $O_2^{\cdot-}$ were detected by lucigenin-enhanced chemiluminiscence, as previously described [10,22]. Cortex samples and 4–6 segments of the renal interlobar arteries about 4–5 mm long from each patient or Wistar rat were dissected and equilibrated in PSS for 30 min at room temperature and then incubated in the absence (controls) and presence of the NADPH oxidase inhibitors plumbagin (1 μM), Nox2ds-tat (1 μM) and GKT136901 (0.1 μM) for 30 min at 37 °C, and then stimulated with NADPH (100 μM) which was added 15 min previous to ROS measurements. Samples were then transferred to microtiter plate wells containing 5 μM bis-N-methylacridinium nitrate (lucigenin) in the absence and presence of different ROS inhibitors. Chemiluminescence was measured in a luminometer (BMG Fluostar Optima), and for calculation baseline values were subtracted from the counting values under the different experimental conditions and superoxide production was normalized to dry tissue weight.