Differential contribution of Nox1, Nox2 and Nox4 to kidney vascular oxidative stress and endothelial dysfunction in obesity

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ARTICLE INFO

Keywords:
Endothelial dysfunction
Renal arteries
Oxidative stress
Nox1
Nox4
Nox2
Obesity

Abstract

Oxidative stress-associated endothelial dysfunction is a key pathogenic factor underlying the microvascular complications of metabolic disease. NADPH oxidase (Nox) is a major source of oxidative stress in diabetic nephropathy and chronic kidney disease, despite Nox4 and Nox2 have been identified as relevant sources of vasodilator endothelial H2O2. The present study was sought to investigate the role of Nox enzymes in renal vascular oxidative stress and endothelial dysfunction in a rat model of genetic obesity. Endothelial function was assessed in intrarenal arteries of obese Zucker rats (OZR) and their counterparts lean Zucker rats (LZR) mounted in microvascular myographs, and superoxide (O2−) and H2O2 production were measured. Impaired endothelium-dependent relaxations to acetylcholine (ACh) were associated to augmented O2− generation, but neither ROS scavengers nor the Nox inhibitor apocynin significantly improved these relaxant responses in renal arteries of OZR. Whereas NO contribution to endothelial relaxations was blunted, catalase-sensitive non-NO non-prostanoid relaxations were enhanced in obese rats. Interestingly, NADPH-dependent O2− production was augmented while NADPH-dependent H2O2 generation was reduced, and cytosolic and mitochondrial SOD were up-regulated in kidney of obese rats. Nox4 was down-regulated in renal arteries and Nox4-dependent H2O2 generation and endothelial relaxation were reduced in OZR. Up-regulation of both Nox2 and Nox1 was associated with augmented O2− production but reduced H2O2 generation and blunted endothelial Nox2-derived H2O2-mediated in obese rats. Moreover, increased Nox1-derived O2− contributed to renal endothelial dysfunction in OZR. In summary, the current data support a main role for Nox1-derived O2− in kidney vascular oxidative stress and renal endothelial dysfunction in obesity, while reduced endothelial Nox4 expression associated to decreased H2O2 generation and H2O2–mediated vasodilatation might hinder Nox4 protective renal effects thus contributing to kidney injury. This suggests that effective therapies to counteract oxidative stress and prevent microvascular complications must identify the specific Nox subunits involved in metabolic disease.

1. Introduction

Oxidative stress is key pathogenic factor underlying the vascular complications of metabolic disease including diabetes- and obesity-related nephropathy and leading to the development and progression of kidney injury [1,2]. Both mitochondria and NADPH oxidases (Nox) are accepted as major sources of ROS generation in diabetic nephropathy and chronic kidney disease, but the specific role of the various Nox subunits in kidney injury remains controversial, since certain Nox enzymes have been implicated in both physiological and pathological renal processes. Thus, Nox2 participates in renal tubular functions such as electrolyte transport and glucose handling [3], while both Nox2 and Nox4 have recently been shown to be sources of endothelium-derived vasodilator H2O2 in renal arteries [4]. On the other hand, Nox4 or renox, the most abundantly expressed Nox isoform in the kidney, has consistently been found up-regulated and associated to kidney fibrosis in diabetes, therefore being proposed as the most critical Nox isoform linked to diabetic nephropathy [5–8]. In contrast, other studies have

https://doi.org/10.1016/j.redox.2019.101330
Received 10 July 2019; Received in revised form 27 August 2019; Accepted 16 September 2019
Available online 20 September 2019

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2. Materials and methods

2.1. Animal model

In the present study, 8–10 weeks of age Male obese Zucker rats (OZR) (fa/fa) and their control counterparts, lean Zucker rats (LZR) (fa/-) were purchased from Charles River RMS (Spain). Rats were housed at the Pharmacy School animal care facility and maintained on standard chow and water ad libitum, until they were used for study, at 17–18 weeks of age. 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, NaHCO3 25, KCl 4.7, KH2PO4 1.17, MgSO4 1.18, CaCl2 1.5, EDTA 0.027 and glucose 11, continuously gassed with a mixture of 5% CO2/95% O2 to maintain pH at 7.4. On the day of the experiment, blood samples were obtained and plasma was frozen for determination of non-fasting glucose, cholesterol, triglycerides and insulin plasma levels by using commercially available kits. Plasma insulin levels were measured by specific enzyme-linked immunosorbent assay.

2.2. Dissection and mounting of microvessels

Renal interlobar arteries, second- or third order branches of the renal artery from LZR and OZR, were carefully dissected by removing the medullary connective 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, L100 corresponding to a transmural pressure of 100 mmHg for a relaxed vessel in situ was calculated. The arteries were set to an internal diameter L1 equal to 0.9 times L100 (L1 = 0.9 x L100), since force development in intrarenal arteries is close to maximal at this internal lumen diameter.

2.3. 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) in the absence and presence of the SOD scavenger (PSS) of the following composition (mM): NaCl 119, NaHCO3 25, KCl 4.7, KH2PO4 1.17, MgSO4 1.18, CaCl2 1.5, EDTA 0.027 and glucose 11, continuously gassed with a mixture of 5% CO2/95% O2 to maintain pH at 7.4. On the day of the experiment, blood samples were obtained and plasma was frozen for determination of non-fasting glucose, cholesterol, triglycerides and insulin plasma levels by using commercially available kits. Plasma insulin levels were measured by specific enzyme-linked immunosorbent assay.

Table 1

| Parameter | LZR | OZR | n |
|-----------|-----|-----|---|
| Body weight (g) | 388 ± 7 | 458 ± 7 | 47 |
| [Glucose]plasma (mg/dl) | 112 ± 10 | 139 ± 10 | 11 |
| [Insulin]plasma (ng/ml) | 1.1 ± 0.1 | 3.93 ± 0.45 | 15 |
| [Cholesterol]plasma (mg/dl) | 89 ± 4 | 184 ± 15 | 10 |
| [Urea]plasma (mg/dl) | 84 ± 9 | 310 ± 40 | 10 |

Data are means ± SEM of the number n of animals. Significant differences were analyzed by an unpaired Student t-test. *p < 0.05; **p < 0.001; ***p < 0.0001 vs LZR.
MitoTEMPO (1 μM), the non-selective Nox inhibitor apocynin (30 μM), catalase (200 IU/mL) and L-NOARG (100 μM) and previously incubated with L-NOARG and indomethacin (1 μM) to block NOS and COX enzymes, respectively. The responses to exogenous ACh were further obtained and compared after treatment with the non-selective Nox4 inhibitor plumbagin (1 μM), the dual Nox1/4 inhibitor GKT137831 (0.1 μM), the selective Nox2 inhibitor Nox2ds-tat (1 μM) and the selective Nox1 inhibitor NoxA1ds-tat (0.1 μM) [26]. 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.

2.4. Measurement of superoxide production by chemiluminescence

Changes in basal, ET-1-induced and NADPH-stimulated levels of O$_2^-$ were detected by lucigenin-enhanced chemiluminescence, as previously described [4,23]. Cortex samples and 4–6 segments of the renal interlobar arteries about 4–5 mm long were dissected out from the kidney of LZR and OZR, equilibrated in PSS for 30 min at room temperature and then incubated in the absence (controls) and presence of the tempol (100 μM) to determine basal O$_2^-$ levels, and in the presence of the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu) (30 μM) and of the vasoconstrictor peptide endothelin (ET-1) (0.01 μM) to measure ET-1-stimulated O$_2^-$ levels, that are greatly enhanced and contribute to vascular oxidative stress and endothelial dysfunction in obesity [27]. To assess Nox-stimulated O$_2^-$ generation, intact intrarenal arteries and cortex samples were incubated with extracellular NADPH (100 μM) for 30 min at 37 °C. Since CYP 2C epoxygenases also contribute to NADPH-dependent O$_2^-$ production in renal tissues [4], treatment with the selective Nox inhibitors Nox2ds-tat (1 μM), GKT137831 (0.3 μM) and NoxA1ds-tat (0.3 μM), was used to determine the relative contribution of the different Nox subunits to ROS generation in renal arteries and cortex from obese rats compared to controls. 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 and of stimulation with NADPH which was added previous to determination. 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.

2.5. Measurement of hydrogen peroxide by Amplex Red

Nox-derived H$_2$O$_2$ levels were measured by Amplex Red H$_2$O$_2$ assay Kit (Life Technologies) in renal arteries and cortex from LZR and OZR. Since apocynin is a non-selective Nox inhibitor that has additional peroxide scavenging properties besides its inhibitory action on Nox activity [28], selective Nox inhibitors were used in order to determine the relative contribution of Nox subunits to H$_2$O$_2$ production in renal tissues. Samples were equilibrated in HEPES-physiological saline solution (PSS) for 30 min at room temperature and then incubated with
NADPH (100μM) in the absence (controls) and presence of the Nox inhibitors Nox2ds-tat (1μM), GKT137831 (0.3μM), Nox1ds-tat (0.3μM) and apocynin (30μM) for 30 min at 37°C. Arteries and cortex samples were then transferred to microtiter plate black wells containing 10mM final concentration (Amplex Red) and 10 U/ml final concentration (horseradish peroxidase) in the absence and presence of different ROS sources inhibitors and some samples were stimulated with either NADPH just prior to determination. Fluorescence was measured in a fluorimeter (BMG Fluostar Optima), using an excitation filter of 544nm and an emission filter of 590nm. Background fluorescence was subtracted from the counting values under the different experimental conditions and H₂O₂ production was normalized to dry tissue weight.

2.6. Western blotting analysis of Nox subunits

Interlobar arteries and renal cortex samples from LZR and OZR were homogenized on ice in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate and 0.01% protease inhibitor cocktail (Sigma Aldrich, Madrid, Spain). After centrifugation at 15000×g for 20 min at 4°C, proteins in the supernatants were quantified by the DC Protein Assay Kit (Bio-Rad, Madrid Spain). For each sample, 50μg protein/lane was separated in a 10% polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Madrid, Spain). For immunodetection, membranes were incubated overnight at 4°C with the polyclonal primary antibodies: anti-CuZnSOD, anti-MnSOD (1/1000), anti-catalase (1/1000) and anti-Nox4 (1/500) (Santa Cruz Biotechnology, Quimigen, Madrid, Spain), anti-Nox1 (1/500) and Nox2/gp91phox (1/1000) (Abcam, Cambridge, UK). The blots were also probed for β-actin (1/40000) as the loading control using a mouse monoclonal antibody (Sigma Aldrich, Madrid, Spain). After washed, bound primary antibody was detected with horseradish peroxidase conjugated secondary antibodies and blots were developed by chemiluminescence (ECL Select-kit, GE Healthcare, Madrid, Spain) on ImageQuant LAS 500 imaging system (GE Healthcare, Madrid, Spain). Densitometry analysis was performed using Quantity One v4.62 software. Protein expression levels were normalized to β-actin.

2.7. Immunohistochemistry

Tissue samples from kidneys containing interlobar arteries and samples of renal cortex from LZR and OZR were immersion-fixed in 4% paraformaldehyde in 0.1M sodium phosphate-buffer (PBS). Kidney paraffin embedded sections (4μm) were deparaffinized and blocked for 10 min with 0.3% hydrogen peroxide at room temperature. After antigen retrieval and blocking, sections were incubated overnight at 4°C with the following biotinylated primary antibodies: anti-CuZnSOD (1/200); anti-MnSOD (1/200); anti-catalase (1/200); anti-Nox1 (1/100); anti-Nox2/gp91phox (1/100) and anti-Nox4 (1/100). Immunohistochemical staining was performed using streptavidin-biotin conjugated horseradish peroxidase (HRP) (EMD Millipore Corp., Darmstadt, Germany) and visualised by incubation with 3, 3’-diaminobenzidine (DAB) (Sigma Aldrich, Madrid, Spain). The sections were counterstained by Harris’s hematoxylin, dehydrated and mounted. Colocalization of Nox4 with eNOS in renal arteries wall was obtained in 5μm thick transversal cryostat sections preincubated in 10% normal goat serum in PB containing 0.3% Triton-X-100 for 2–3 h. Nox4 expression was determined...
by immunofluorescence by incubating renal sections from LZR and OZR with a polyclonal primary antibody anti-Nox4 (1:100) (Santa Cruz Biotechnology, Quimigen, Madrid, Spain) and a mouse monoclonal anti-eNOS (Abcam, Cambridge, UK) diluted at 1:400 for 48h, washed and allowed to react with a goat secondary serum (Chemicon International Inc) (anti-rabbit for the Nox4) diluted 1:200 for 3 h at room temperature. Secondary antibodies used were Alexa Fluor 594 (red) and Alexa Fluor 488 (green). The slides were covered with a specific medium containing DAPI, which stains all cell nuclei. The observations were made with a fluorescence microscope (Olympus IX51). No immunoreactivity could be detected in sections incubated in the absence of the primary antiserum. Preadsorption with Nox proteins showed no cross-reactivity to the antibodies.

2.8. Data presentation and statistical analysis

For the functional experiments, results are expressed as either Nm\(^{-1}\) or tension or as a percent of the responses to either Phe or KPSS in each artery, as means ± SEM of 6–15 arteries (1–2 from each animal). For measurements of \(O_2^-\) or \(H_2O_2\) production, results are expressed as counts per minute (cpm) per mg of tissue and relative fluorescence units (RFU) per mg of tissue in arterial segments and cortex samples, respectively, as means ± SEM of 4–7 animals. The statistical differences between means were analyzed by using one-way ANOVA followed by Bonferroni’s post hoc test for comparisons involving more than two groups or by paired or unpaired Student’s t-test for comparison between two groups. Probability levels lower than 5% were considered significant. All calculations were made using a standard software package (Prism 6.0, GraphPad, San Diego, CA).

3. Results

3.1. General parameters

Metabolic profile and plasma renal function parameters are shown in Table 1. At the time of the experiment (17–18 weeks of age), OZR were significantly heavier than LZR and exhibited mild hyperglycemia, hyperinsulinemia and dyslipidemia with elevated total cholesterol and triglycerides levels. Renal function, as assessed by plasma creatinine and urea, remained normal (Table 1).

The normalized internal lumen diameters, \(l_1\), of renal arteries in the OZR group (252 ± 9 μm, \(n = 30\)) were not significantly different from those in the LZR group (247 ± 14 μm, \(n = 23\)), thus supporting that structure is preserved in arteries from OZR compared to LZR. Vasoconstrictor response to KPSS (1.18 ± 0.18 Nm\(^{-1}\), \(n = 23\), in LZR and 1.18 ± 0.11, \(n = 30\), in OZR) or Phe (1.31 ± 0.20 Nm\(^{-1}\), \(n = 23\), in LZR, and 1.28 ± 0.17, \(n = 30\), in OZR) were also unchanged in OZR.

3.2. Oxidative stress and endothelial dysfunction resistant to antioxidants in renal arteries of OZR

Basal \(O_2^-\) levels assessed by lucigenin chemiluminescence, and also those stimulated by ET-1 or PDBu, were significantly higher in renal arteries of OZR compared to LZR (Fig. 1a), thus showing vascular oxidative stress in the kidney of obese animals. Endothelial function was evaluated by the vasodilator responses to acetylcholine (ACh) that were significantly impaired in renal interlobar arteries from obese rats (Fig. 1a). However, the endothelial relaxations elicited by ACh were not improved by acute treatment with either the SOD mimetic tempol (Fig. 1c), the mitochondrial ROS scavenger MitoTEMPO (Fig. 1d) or the non-selective Nox inhibitor apocynin (Fig. 1e) in OZR, despite augmented oxidative stress in renal arteries.

\(H_2O_2\) has recently been involved along with NO in the endothelium-dependent relaxations of intrarenal arteries [4], and therefore the relative contribution of NO and \(H_2O_2\) were determined in arteries from lean and obese animals in order to assess the nature of the impaired ACh relaxant responses. The NO-mediated component of the ACh relaxations, obtained in the presence of catalase (Fig. 2a and b) and enhanced by treatment with indomethacin to block COX-derived contractile prostanoids (Fig. 2c and d), was significantly reduced in arteries of OZR, thus confirming impaired vasodilator responses to NO in obese rats. Interestingly, a marked enhancement of the relaxations induced by ACh was unmasked when assessing endothelial relaxations under conditions of NOS and COX enzymes blockade (Fig. 2e and f), thus suggesting the involvement of compensatory non-NO non-prostanoid EDH type mechanisms in renal arteries from obese rats. However, \(H_2O_2\) did not account for these enhanced relaxations since catalase nearly abolished the non-NO endothelial relaxant component in LZR but not in OZR (Fig. 2e and f).

3.3. Nox-derived \(O_2^-\) production is elevated while Nox-derived \(H_2O_2\) generation is decreased in renal arteries from obese rats

In order to assess the contribution of Nox-derived oxidative stress to renal endothelial dysfunction of obese rats, NADPH-stimulated \(O_2^-\) and \(H_2O_2\) production was measured by lucigenin chemiluminescence and Amplex Red fluorescence, respectively, in the absence and presence of the non-selective NOX inhibitor apocynin. Both \(O_2^-\) and \(H_2O_2\) levels were markedly enhanced by NADPH in both renal interlobar arteries.
and renal cortex (Fig. 3). Although a contribution of CYP epoxygenases to the enhanced NADPH-dependent ROS generation in obese rats cannot be ruled out [4], the marked inhibitory effect of apocynin on O$_2^-$ and to a lesser extent on H$_2$O$_2$ levels, suggests a major contribution of Nox to oxidative stress in renal tissues from OZR. Interestingly, while NADPH-stimulated O$_2^-$ levels abolished by apocynin were significantly larger in arteries, and to a lesser extent in renal cortex, of OZR compared to LZR (Fig. 3a and b), NADPH-stimulated H$_2$O$_2$ generation was significantly reduced in both arteries and cortex of obese rats compared to lean controls (Fig. 3c and d). Basal H$_2$O$_2$ levels were also significantly lower in renal cortex of obese rats. These data indicate that augmented Nox-derived O$_2^-$ generation contributes renal vascular oxidative stress, whereas kidney Nox-derived H$_2$O$_2$ production was decreased in obesity.

3.4. Up-regulation of vascular antioxidant defences in OZR

Vascular oxidative stress measured as increased O$_2^-$ generation in renal interlobar arteries was associated with the enhanced expression of the enzymes dismutating O$_2^-$ to H$_2$O$_2$, the cytosolic CuZnSOD (Fig. 4a and b) and the mitochondrial MnSOD (Fig. 4c and d) in the kidney of OZR, as depicted by the Western Blot analysis. Immunostaining of arterial sections further demonstrated that up-regulation of these antioxidant enzymes was particularly evident in VSM of obese rats (Fig. 4a,c). Western blot analysis and immunohistochemistry confirmed that catalase protein levels were also up-regulated and significantly higher in renal arteries from OZR compared to LZR but not in renal cortex (Supplementary Fig. 1).

3.5. Endothelial Nox4 expression, Nox4-derived H$_2$O$_2$ and Nox4-mediated endothelium-dependent relaxations are reduced in obese rats

In order to assess the relative contribution of the different Nox subunits to arterial oxidative stress and renal endothelial dysfunction in obese rats, Nox proteins were determined by Western blot and immunohistochemistry in renal interlobar arteries and cortex, and the effect of selective Nox inhibitors were evaluated on ROS production and on the H$_2$O$_2$-mediated endothelium-dependent relaxations of renal arteries [4].

Endothelial Nox4 has recently been identified as a physiological source of vasodilator H$_2$O$_2$ in renal arteries. Immunostaining of arterial sections showed that Nox4 was colocalized with eNOS protein in the endothelium of renal arteries from LZR (Fig. 5a and c), while Nox4 expression was reduced in the endothelium but it was found in VSM in OZR (Fig. 5a and c). No apparent differences in either distribution or density of the Nox4 immunolabeling were observed between LZR and OZR in renal cortex (Fig. 5b). Western blot analysis confirmed that total Nox4 protein levels were significantly reduced in renal arteries from obese rats compared to controls (Fig. 5d), while they were similar in cortical tissue of LZR and OZR (Fig. 5e).

The effect of the selective Nox1/4 inhibitor GKT137831 was next examined on ROS production of intrarenal arteries. GKT137831 reduced both O$_2^-$ (Fig. 6a) and H$_2$O$_2$ (Fig. 6b) formation stimulated by NADPH in LZR and OZR. This compound had a larger inhibitory effect on O$_2^-$ production in OZR (Fig. 6a), while the inhibitory effect on H$_2$O$_2$ generation was reduced in obese animals compared to controls (Fig. 6b). To determine whether changes in the Nox4 metabolism may
be involved in the endothelial dysfunction observed in renal arteries of OZR, the effect of GKT137831 and of the non-selective Nox4 inhibitor plumbagin was assessed on the endothelium-dependent relaxations elicited by ACh. These relaxant responses were significantly inhibited by GKT137831 and by plumbagin in LZR (Fig. 6c and e) thus confirming the involvement of endothelial Nox4-derived H$_2$O$_2$ [4], but this inhibition was lesser in lean rats (Fig. 6d and f).

3.6. Up-regulation of arterial Nox2 is associated to reduced endothelial Nox2-derived H$_2$O$_2$-mediated relaxations

Nox2-derived H$_2$O$_2$ is also involved in the endothelium-dependent relaxations of renal arteries [4] and its role in renal endothelial dysfunction of obese rats was assessed. Immunoreaction for Nox2 was found in interlobar arteries from LZR and OZR (Fig. 7a), while in renal cortex, Nox2 was widely distributed in tubular structures in LZR but its expression was markedly reduced in OZR (Fig. 7c). Western blot analysis showed higher Nox2 protein levels in rat intrarenal arteries of OZR compared to LZR (Fig. 7b), while there was a pronounced down-regulation of Nox2 in renal cortex of obese animals (Fig. 7d). The selective inhibitor of Nox2 Nox2ds-tat abolished O$_2^-$ production stimulated by NADPH in renal interlobar arteries of both LZR and OZR (Fig. 8a) and reduced H$_2$O$_2$ generation in LZR but hardly affect H$_2$O$_2$ production in OZR (Fig. 8b). To assess whether changes in Nox2-derived ROS may also contribute to renal endothelium dysfunction in OZR, renal arteries of LZR and OZR were treated with the selective Nox2 inhibitor Nox2ds-tat, which significantly reduced the relaxant responses in lean (Fig. 8c) but did not further affect these responses in renal arteries of obese rats (Fig. 8d).

3.7. Nox1 rather than Nox4 preferentially contributes to kidney vascular oxidative stress and endothelial dysfunction in obesity

Nox1 has recently been proposed to be the member of the NADPH oxidase family responsible for microvascular dysfunction in metabolic disease [29]. Nox1 protein was determined by immunohistochemistry and Western Blot in the kidney of LZR and OZR. Immunostaining of arterial samples with Nox1 antibodies revealed a very low expression of this subunit in renal arteries and cortex of LZR, but obesity markedly enhanced kidney Nox1 expression (Fig. 9a and c). Nox1 was found in VSM of renal arteries, and in both renal tubules and vascular tissue - glomeruli and arterioles- in samples of renal cortex of obese rats (Fig. 9a and c). Western Blot analysis confirmed that Nox1 protein levels were significantly up-regulated in isolated renal arteries (Fig. 9b) and renal cortex (Fig. 9d), of obese animals compared to controls.

Augmented NADPH-stimulated O$_2^-$ in renal arteries from OZR were
Oxidative stress has been implicated in the pathogenesis of diabetes and obesity-related microvascular complications including kidney injury [1,2,30]. The major findings of the present study are that augmented Nox-derived O$_2^-$ is involved in kidney vascular oxidative stress and endothelial dysfunction in obesity but there is a differential contribution of the various Nox members of the NAPDH oxidase family. While Nox1 and Nox2 were up-regulated in renal arteries and contributed to the increased O$_2^-$ generation and endothelial dysfunction, reduced endothelial Nox4 expression was associated with decreased Nox-derived H$_2$O$_2$ and impaired H$_2$O$_2$-mediated vasodilatation which might hinder Nox protective renal vascular effects and thus indirectly promote impaired endothelial function.

Renal inflammation and oxidative stress have earlier been found along with endothelial dysfunction in renal arterioles of genetic and HFD models of obesity [18,23], and initially ascribed to up-regulation of COX-2 that contributed to plasma and renal oxidative stress and augmented vasoconstriction [23,31]. The present data confirm endothelial dysfunction in renal interlobar arterioles of genetically obese rats, as depicted by the impaired ACh-induced relaxations; however, antioxidant treatment including non-selective Nox inhibition hardly ameliorated impaired ACh relaxant responses despite augmented basal and ET-1-stimulated O$_2^-$ levels in the arterial wall, that have been reported to greatly contribute to vascular oxidative stress and reduced endothelial NO bioavailability in obesity [27]. A substantial fraction of the endothelium-dependent responses of renal arterioles is mediated by a non-NO non-prostanoid relaxing factor and is associated with VSM hyperpolarization [32,33]. When pharmacologically dissecting the components of the relaxant responses of renal arteries in OZR, we found that enhanced non-NO non-prostanoid EDH type relaxations compensated for impaired NO-mediated responses of renal arteries in obese rats, as depicted by the lesser reduction of these relaxations by NOS inhibition in OZR, but the larger relaxant responses unmasked upon blockade of NO and prostanoit synthesis. This increased contribution of EDH-type responses to counteract diminished NO bioavailability is consistent with that recently shown in coronary, mesenteric and subcutaneous arteries from diabetic rats and patients [34–36], and ascribed to the enhanced activity of endothelial K$_{Ca}$ channels [35]. On the other hand, impaired NO-mediated endothelial dysfunction found for renal interlobar arteries of obese rats would also be expected to occur in different arterioles, wherein the relative contribution of NO and non-NO non prostanoit EDH-type factors has earlier been reported to be similar to that found for the preglomerular arteries in the present study [33].

H$_2$O$_2$ derived from both CYP 2C epoxygenases and Nox contributes to the EDH-type responses in healthy renal arterioles [4,37], which might explain the lack of significant effect of ROS scavengers on endothelial relaxations of OZR despite augmented arterial oxidative stress. However, the enhanced EDH-type relaxant responses observed in renal arteries from obese rats cannot be ascribed to a larger contribution of H$_2$O$_2$ to these responses since they were not further inhibited by catalase. Moreover, NADPH-dependent H$_2$O$_2$ generation was reduced in both renal arteries and cortex of OZR compared to lean controls despite the overall NADPH-dependent O$_2^-$ production was enhanced in kidney tissues, in particular in intrarenal arteries. Diminished H$_2$O$_2$ levels cannot either be due to reduced antioxidant H$_2$O$_2$-generating enzymes CuZnSOD and MnSOD, since they were up-regulated in both renal arteries and cortex, in particular the mitochondrial SOD isoform whose expression was markedly enhanced in vascular tissue including renal arterioles and glomeruli. Interestingly, these results are in line with those recently reported for the diabetic kidney wherein reduced mitochondrial H$_2$O$_2$ generation measured with Amplex Red was observed despite the enhanced overall kidney ROS production, suggesting an

Fig. 6. The Nox4 inhibitors GKT137831 and plumbagin reduce ROS generation and endothelial H$_2$O$_2$-mediated relaxations in renal arteries of lean and to a lesser extent of obese rats. (a,b) Effect of the Nox1/4 inhibitor GKT137831 (0.3 μM) on the NADPH-stimulated levels of O$_2^-$ (a) and H$_2$O$_2$ (b) measured by lucigenin-enhanced chemiluminescence and by Amplex Red fluorescence, respectively, in renal arteries of LZR and OZR. Results are expressed in counts per minute (cpm) per mg of tissue for chemiluminescence and in relative fluorescence (RFU) per mg of tissue for fluorescence. Bars represent mean ± SEM of 3–10 animals. Significant differences between means were analyzed using one-way ANOVA followed by Bonferroni as a posteriori test. ***p < 0.001 versus basal levels, †p < 0.05; †††p < 0.001 versus NADPH-stimulated, ##p < 0.01 versus LZR. (c–f) Average inhibitory effect of the Nox1/4 GKT137831 inhibitor (0.3 μM) (a,f) and the non-selective Nox4 inhibitor plumbagin (1 μM) (e,f) on the relaxations induced by the endothelium agonist ACh under conditions of NOS and COX blockade (100 μM L-NOARG and 0.3 μM indomethacin) in renal arteries of LZR (c,e) and OZR (d,f). Data are shown as the mean ± SEM of 6–13 arteries from 7 animals. Significant differences were analyzed using Student t-test for paired observations *p < 0.05; **p < 0.01 versus control treatment.

significantly reduced by the selective Nox1 inhibitor NoxA1ds (Fig. 10a), and also H$_2$O$_2$ levels of LZR and to a lesser extent those of OZR (Fig. 10b). The effect of the selective Nox1 inhibitor was further evaluated on the relaxant responses to the endothelial agonist ACh under conditions NOS and COX blockade. NoxA1ds hardly affected ACh-elicited relaxations in LZR (Fig. 10c) and those in OZR (Fig. 10d), thus ruling out a major role of Nox1-derived H$_2$O$_2$ in the endothelium-dependent vasodilatation of renal arteries. However, in the absence of L-NOARG to inhibit eNOS and NO formation, selective inhibition of Nox1 with NoxA1ds significantly improved impaired ACh endothelial relaxations in OZR thus suggesting that Nox1-derived O$_2^-$ contributes to renal endothelial dysfunction in obesity.

4. Discussion
impaired mitochondrial activity that was ascribed to AMPK dysregulation [2,38]. Interestingly, in our study a reduced expression of Nox4, mostly restricted to endothelial cells in healthy arteries [4], was also found along with the diminished NADPH-dependent H$_2$O$_2$ generation in renal arteries of obese rats suggesting that down-regulation of this Nox subunit might be involved in the lower levels of arterial H$_2$O$_2$. Moreover, Nox4-derived H$_2$O$_2$ production measured with Amplex Red and to a lesser extent its contribution to the EDH-type endothelial relaxations of renal arterioles from OZR were also significantly reduced, as depicted by the lower inhibitory effect of the Nox inhibitors GKT137831 and plumbagin on these responses compared to that on arteries from lean rats. Thus, in contrast to Nox1 or Nox2 that primarily produce O$_2^-$, Nox4 produces H$_2$O$_2$ rather than O$_2^-$ [39] and has been identified as a functional source of ROS generation in the mitochondria of kidney cortex, wherein the mitochondrial SOD effectively dismutates Nox4-derived superoxide to H$_2$O$_2$ [40]. Moreover, mitochondria-derived H$_2$O$_2$ has recently been shown to effectively contribute to the EDH-type endothelium-dependent relaxations of renal small arteries [4].

Down-regulation of arterial Nox4 expression and reduced Nox4-derived H$_2$O$_2$ production in genetically obese rats contrast with studies linking Nox4 to renal pathology and showing that genetic deficiency or pharmacological inhibition of Nox4 ameliorated enhanced Nox4-derived ROS generation, glomerular injury and kidney inflammation and fibrosis in diabetic mice [6,7]. However, our results showing lower levels of vascular Nox4 coupled to impaired Nox4-derived H$_2$O$_2$ generation would be consistent with the reduced expression of tubular Nox4 and H$_2$O$_2$ generation found in hyperglycemic type 1 diabetic mice and in other models of chronic kidney disease, as well as with the worsening of renal function and fibrosis upon genetic deletion of Nox4 in diabetic models, which suggests a protective role of Nox4 in renal disease [9,10]. The protective role of endothelial Nox4 as a source of vasodilator H$_2$O$_2$ in renal arteries recently shown by us is also confirmed in our study, but the impact of reduced Nox4-derived endothelial H$_2$O$_2$ generation in renal endothelial function of obese rats is difficult to assess due to the non-NO non-prostanoid EDH-type mechanisms that compensate for the diminished NO bioavailability and impaired endothelial vasodilatation [34–36].

Nox2 and Nox1 have been linked to endothelial and vascular dysfunction in metabolic disease and hypertension [41–45], and both Nox subunits, in particular Nox2, are closely related to vascular inflammation [41]. Many metabolic and cardiovascular disorders including insulin resistance, obesity and atherosclerosis exhibit a chronic low-grade inflammation at the vascular wall and are associated with the redox-sensitive NF-κB inflammatory signaling pathway activated by Nox-derived ROS in response to high levels of glucose, LDL cholesterol and free fatty acid (FFA) stimulation [41]. Since obesity is usually related to other metabolic and vascular disorders such as insulin resistance, dyslipidaemia, impaired glucose tolerance and hypertension, jointly referred to as the metabolic syndrome [46], dyslipidemia (hypertriglyceridemia and hypercholesterolemia) and insulin resistance of the obese Zucker rat used in the present study could in fact account for the marked up-regulation of pro-inflammatory Nox2 and Nox1 subunits.
and enhanced Nox-derived oxidative stress found in renal arteries of obese animals [41,46,47]. Expression of Nox2 (gp91phox), the classic NADPH oxidase primarily found in phagocytic cells, followed different trends in kidney cortex and renal arteries of obese rats. Thus, whereas Nox2 was up-regulated in renal arteries, its expression was markedly reduced in renal cortex of OZR. Up-regulation of arterial Nox2 was followed by gp91ds-tat or siRNA-mediated knockdown of Nox2, while insulin-resistance-induced impaired endothelium-dependent vasodilation and vascular function was improved by Nox2 deletion [49],

Moreover, ex vivo exposure to insulin reduced flow-induced vasodilation and NO formation and increased ROS production in human skeletal muscle arterioles, these insulin effects being reversed by Nox2 inhibitors [50]. In the latter study, hyperinsulinemia increased Nox2 but not Nox4 expression and activity thus augmenting O$_2^-$ generation in microvascular endothelial cells from adipose tissue, which reduces NO availability and enhances peroxynitrite formation [50]. In our study, while O$_2^-$, was enhanced Nox2-derived H$_2$O$_2$ levels were significantly reduced in renal arteries from OZR compared to lean rats, and so were the Nox2-dependent H$_2$O$_2$-mediated endothelium dependent relaxations. Endothelial H$_2$O$_2$ derived from both Nox4 and Nox2 contributes to the endothelium-dependent relaxations of healthy renal arteries [4], and the present data demonstrate that this contribution is impaired in obesity therefore hindering Nox protective effects on renal endothelial function.

Interestingly, in contrast to the enhanced expression of Nox2 in renal arteries, Nox2 was markedly down-regulated in kidney cortex, specifically in renal tubules of obese rats, in agreement with that earlier found in leptin receptor-deficient hyperglycemic db/db mice wherein both Nox2 gen and protein expression were reduced in renal cortex [6]. In the latter rodent model of type II diabetes and obesity, down-regulation of Nox2 was interpreted as a compensatory mechanism to counterregulate the enhanced Nox4/p22phox expression, augmented ROS generation and activation of redox-sensitive proinflammatory pathways being ascribed to Nox4 [6]. However, in our study the whole Nox4 subunit content was unchanged in kidney cortex and markedly reduced in renal arteries of insulin resistant OZR. On the other hand, studies in Nox2-deficient mice have further demonstrated that the lack of Nox2 does not protect against oxidative stress, glomerular hypertrophy and fibrosis in diabetic kidney disease, the latter being again ascribed to up-regulation of Nox4 [12]. In fact, Nox2-derived superoxide is involved in several renal tubular functions, such as regulation of tubuloglomerular feedback and mesangial cell responses to high salt [51] and potassium transport in the collecting duct [52]. Therefore, taken together, the present data along with those reported in the kidney of rodent models of type 2 diabetes showing decreased expression of Nox2 gene and protein [6] suggest that loss of Nox2-mediated renal tubular functions might contribute to diabetes and obesity-associated kidney injury.

Unlike Nox2 and Nox4 members of the NADPH oxidase family, whose expression was either unchanged or downregulated in renal cortex of insulin resistant obese rats, Nox1 was marked and uniformly enhanced in renal cortex of OZR which supports earlier findings in glomeruli of type 1 diabetic mice [53] and cortex of HFD-fed rats [17]. In these studies, Nox1-derived oxidative stress was associated with inflammation, podocyte injury and albuminuria, and treatment with the SOD mimetic tempol improved metabolic profile and ameliorated inflammation, podocyteinjury and albuminuria, and treatment with the SOD mimetic tempol improved metabolic profile and ameliorated kidney injury and renal dysfunction [17], thus suggesting a main role of Nox1 in diabetic and obesity nephropathy. In our study, Nox1 was mostly found in arterioles and renal tubule epithelium of renal cortex, the latter supporting earlier studies showing that Nox1 is highly expressed in the intestinal epithelium in response to transcriptional up-regulation of its promoter by cytokines involved in host defense [41,54]. Many of the renoprotective effects on diabetic nephropathy reported for the Nox1/Nox4 inhibitor and ascribed to Nox4 could therefore be also due to Nox1 [7,11,55].

Nox1 is a major source of O$_2^-$ generation in endothelial cells, VSM cells and fibroblasts under both physiological and pathophysiological conditions. This Nox subunit participates in shear-induced outsowards vascular remodelling, while in vascular pathology it has primarily been involved in hypertension and proliferative vascular disease [41]. Both Nox2 and Nox4, but not Nox1 are expressed in human and rat intrarenal arteries [4,56]. In the present study, a low expression of Nox1 was found in renal arteries of lean rats, but obesity induced a marked up-regulation of this subunit in VSM of both interlobar arteries and cortical arterioles. Recent studies have identified Nox1 as a critical
source of ROS responsible for microvascular dysfunction in metabolic disease, and deletion of Nox1 [29] or the Nox1 organizer NoxO1 [45] abolished oxidative stress and improved endothelial dysfunction and VSM dilation in mesenteric microvessels of diabetic mice, and attenuated atherosclerosis development in diabetes mellitus [41,55]. The present data support the involvement of Nox1 in kidney vascular oxidative stress in obesity, since both O$_2^-$ and H$_2$O$_2$ production were abolished by the selective Nox1 inhibitor NoxA1ds in renal arterioles of OZR. On the other hand, in contrast to Nox2 and Nox4 [4], Nox1 does not seem to be a relevant physiological source of vasodilator H$_2$O$_2$, since Nox1ds did not significantly attenuate non-NO non-prostanoid endothelial relaxations of intrarenal arteries in LZR, although it had a moderate inhibitory effect of these responses in obese rats, possibly due to up-regulation of VSM CuZnSOD activity that dismutates augmented Nox1-derived O$_2^-$ to H$_2$O$_2$ in renal arteries.

In the present study, up-regulation of Nox1 was particularly evident in VSM of obese rats. Overexpression of VSM Nox1 leading to enhanced production of ROS in response to angiotensin II causes eNOS uncoupling and decreases NO bioavailability resulting in impaired vasorelaxation thus showing a crosstalk between Nox1 up-regulation in VSM and endothelial function [57]. Moreover, NoxO1 and p47phox-dependent activation of Nox1, but not that of Nox2, Nox4 or mitochondrion, mediates eNOS uncoupling and endothelial dysfunction in type I diabetic mice [58]. Augmented basal oxidative stress in renal interlobar arteries of obese rats is in part due to eNOS uncoupling and reduced by NOS blockade [23]. The present data show that the absence of NOS blockade unmasked a beneficial effect of the selective Nox1 inhibitor NoxA1ds on the impaired NO-mediated relaxations of renal arteries of genetically obese rats. This improvement was not observed with apocynin, a general Nox inhibitor with higher affinity for Nox2, which suggests that Nox1-derived O$_2^-$ mostly derived of VSM impairs NO-mediated endothelium-dependent responses and is therefore involved in renal endothelial dysfunction in obesity.

In conclusion, the present study provides novel data on the differential contribution of Nox subunits from the NADPH family to kidney vascular oxidative stress and endothelial dysfunction in obesity. Nox4, considered to play a key role in the pathogenesis of diabetic nephropathy, is down-regulated in the renal endothelium of obese rats and the contribution of Nox4-derived H$_2$O$_2$ to renal endothelial vasodilation is impaired, thus hindering Nox4 protective vascular effects. In contrast, Nox1 is uniformly up-regulated in both arterial and renal cortex tissues of obese rats and Nox1-derived O$_2^-$ underlies endothelial dysfunction, which suggests that this specific Nox subunit might be critical in the development of obesity-associated microvascular complications.
Sources of support and vasculardysfunction in obesity-related nephropathy. Of selective Nox1 inhibitors maybe beneficial to prevent endothelial damage effect might have indeed been due to Nox1 inhibition, and the use of selective Nox1 inhibitors may be beneficial to prevent endothelial and vascular dysfunction in obesity-related nephropathy.

Conflicts of interest

All the authors declare no conflicts of interest.

Acknowledgements

We thank Francisco Puente and Manuel Perales for their expert technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101330.

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Appendix A. Supplementary data

This work was supported by grant SAF2016-77526R from Ministerio de Economía y Competitividad (MINECO, Spain) Fondo Europeo de Desarrollo Regional (FEDER).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101330.
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