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Angiotensin II Reduces Transport-Dependent Oxygen Consumption but Increases Transport-Independent Oxygen Consumption in Immortalized Mouse Proximal Tubular Cells

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Abstract

Oxidative stress is closely associated with renal dysfunction following diabetes and hypertension. Angiotensin II (Ang II) can activate the NADPH- oxidase, increasing oxidative stress that is thought to blunt proximal tubular electrolyte transport and thereby oxygen consumption (QO_2). We investigated the effect of Ang II on QO_2 in immortalized mouse proximal tubular cells over-expressing the NADPH oxidase subunit $p22^{phox}$; a model of increased oxidative stress. Cultured cells were exposed to either Ang II or H_2O_2 for 48 h. QO_2 was determined during baseline (113 mmol/l NaCl; transport-dependent QO_2) and during sodium-free conditions (transport-independent QO_2). Ang II reduced transport-dependent QO_2 in wild-types, but not in $p22^{phox}$ which also displayed increased QO_2 at baseline. Transport-independent QO_2 was increased in $p22^{phox}$ and Ang II had no additional effect, whereas it increased QO_2 in wild-type. Addition of H_2O_2 reduced transport- dependent QO_2 in wild-types, but not in $p22^{phox}$. Transport-independent QO_2 was unaffected by H_2O_2. The similar effects of Ang II and H_2O_2 to reduce transport-dependent QO_2 suggest a direct regulatory role of oxidative stress. In accordance, the transport-dependent QO_2 was reduced in $p22^{phox}$ already during baseline. The effects of Ang II on transport-independent QO_2 was not replicated by H_2O_2, indicating direct regulation via Ang II-receptors independently of oxidative stress. However, the Ang II effect was absent in $p22^{phox}$,

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suggesting that oxidative stress also modulates normal Ang II signaling. In conclusion, Ang II affects both transport-dependent and transport-independent QO_2 in proximal tubular cells and may be an important pathway modulating renal QO_2.

Keywords

Proximal tubule cell; Oxidative stress; Angiotensin-II; Oxygen consumption; Electrolyte transport

1 Introduction

Development of nephropathy due to diabetes and hypertension is strongly connected with concurrent development of renal hypoxia [1]. Renal hypoxia may develop with increased metabolic demand, renal anemia, or when renal peritubular capillary blood flow is decreased due to glomerular injury or vasoactive substances constricting the arterioles. Angiotensin II (Ang II), a vasoactive substance known to be increased in both hypertensive and diabetic kidneys [2, 3], induces oxidative stress and constriction of afferent as well as efferent arteriole [4]. Both these effects reduce renal blood flow and therefore reduce oxygen delivery, contributing to renal hypoxia. The metabolic demand by the kidney is largely determined by electrolyte reabsorption, accounting for 80 % of total kidney oxygen consumption (QO_2) [5]. Interestingly, the energy-demanding Na^+/K^+-ATPase-activity is increased in diabetes [6], increasing QO_2 and possibly limiting renal oxygenation. Diabetes-induced increased QO_2 and development of renal hypoxia are closely linked to increased levels of oxidative stress, as demonstrated by the prevention of renal hypoxia using antioxidants [7]. Interestingly, Ang II activates the NADPH oxidase via the Ang II receptor subtype 1 (AT1-R), resulting in increased superoxide formation [8] and several studies have suggested a role for NADPH-oxidase in the development of both hypertension and nephropathy [9, 10].

In summary, renal QO_2 is affected by mitochondrial activity, electrolyte transport and cellular QO_2, all processes that are altered by oxidative stress. However, the detailed pathways of Ang II-mediated effects on proximal tubular QO_2 and their relation to increased oxidative stress are presently unknown. Therefore, the present study separated the effects of Ang II on electrolyte transport-dependent QO_2 from those on transport-independent QO_2 in immortalized wild-type and $p22^{phox}$ overexpressing mouse proximal tubular cells. The latter is a model of increased oxidative stress and was utilized to separate the effects of AT1-R signaling *per se* from those of Ang II-induced oxidative stress.

2 Material and Methods

Immortalized proximal tubular cells with and without a stable over-expression of the NADPH oxidase subunit $p22^{phox}$ were maintained at 37°C and 5 % CO_2 in Dulbecco's Modified Eagle Medium/F12 medium containing 5 % fetal bovine serum. At 50 % confluency, cell splitting was routinely performed using 2.5 % trypsin. Both cell types were grown with or without Ang II (10^{-7} mol/l, Sigma-Aldrich, St Louis, MO, USA replaced every 12 h) and H_2O_2 (2.5×10^{-5} mol/l, Sigma-Aldrich, St Louis, MO, USA replaced every 12 h) for 48 h. Before measurements, cells were placed in suspension with 2.5 % trypsin

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followed by triple rinsing by slow centrifugation (100 × *g*, 4 min, 4°C) in ice-cold respiration buffer (in mmol/l: glucose 23.2; NaCl 113; KCl 4.0; $NaHCO_3$ 27.2; KH_2PO_4 1.0; $MgCl_2$ 1.2; $CaCl_2$ 1.0; HEPES 10.0; Ca^{2+}-lactate 0.5; glutamine 2.0 and streptomycin 50 U/ml; osmolality 298 ± 2 mOsm checked with a freezing point osmometer, Fiske laboratories; pH 7.4) with or without Na^+. In absence of NaCl and $NaHCO_3$, osmolality was kept constant with 280.4 mOsm mannitol. The cell-suspension was kept on ice until QO_2 was measured as described previously [11]. Briefly, a custom-made 1.1 ml gas-tight Plexiglas chamber thermostatically controlled at 37°C with continuous stirring from an air driven magnetic stirrer was used to determine QO_2 on free-floating cells in respiration buffer with or without Na^+ to evaluate transport-dependent QO_2 and transport-independent QO_2 respectively. QO_2 was determined as rate of oxygen disappearance as measured by a modified Unisense-500 O_2-sensor (Unisense A/S, Aarhus, Denmark) calibrated with the air-equilibrated buffer solution as 228 μmol/l O_2 and $Na_2S_2O_5$-saturated buffer as zero, and normalized to protein concentration. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Multiple comparisons between groups were performed using analysis of variance (ANOVA) followed by Sidak multiple comparisons test. All data are presented as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant.

3 Results

Cells overexpressing $p22^{phox}$ had reduced transport-dependent QO_2 compared to wild-type. Ang II reduced transport-dependent QO_2 in wild-type but not in $p22^{phox}$. During baseline conditions, transport-independent QO_2 was increased in $p22^{phox}$ compared to wild-type. Ang II displayed only a tendency to decrease transport- dependent oxygen consumption in $p22^{phox}$ whereas it increased transport- independent QO_2 in wild-type (Fig. 21.1). Addition of H_2O_2 reduced transport-dependent QO_2 in wild-type but only tended to reduce QO_2 in $p22^{phox}$. However, transport-independent QO_2 was unaffected by H_2O_2 in both cell types (Fig. 21.2).

4 Discussion

The present study demonstrates a role for Ang II in regulating transport-dependent as well as transport-independent QO_2 in mouse proximal tubular cells. It is likely that Ang-II decreases transport-dependent QO_2 by inducing oxidative stress, a hypothesis strengthened by the fact that the effect is mimicked by elevating oxidative stress via H_2O_2. Furthermore, the response to Ang II is reduced in cells with increased levels of oxidative stress during baseline, i.e. the $p22^{phox}$, further highlighting oxidative stress as a crucial component in the mechanism of Ang II.

However, as the effects on transport-independent QO_2 is not mimicked by H_2O_2 other mechanisms than oxidative stress may be involved.

It is known that Ang II increases superoxide formation by activating NADPH oxidase via AT-1R [8, 12]. Indeed, inhibition of AT-1R by olmesartan reduces oxidative stress independently of its blood pressure-lowering effect [13]. Several studies have suggested a

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role for NADPH oxidase in the development of hypertension and kidney disease [10, 14]. Oxidative stress is indeed increased in human hypertension [15] as well as in several hypertensive animal models [16]. AT_1-Receptor blockade with candesartan has been reported equally effective as the superoxide dismutase mimetic tempol in normalizing renal pO_2, implying that oxidative stress is directly involved [17]. Furthermore, inhibition of Ang II with angiotensin- converting enzyme (ACE) inhibitors and AT1-R blockers lowers renal QO_2 [18]. Ang II acting on AT_1-Receptors has been implied for the development in intrarenal hypoxia during hypertension, since 2 weeks of treatment with the AT1-R blocker candesartan to SHR rats normalize renal pO_2 [19]. Finally, Ang II-dependent hypertension in rats increases transport-dependent QO_2 in the thick ascending limb [20].

Cells with increased levels of oxidative stress displayed increased transport- independent QO_2. This may be due to mitochondrial uncoupling by uncoupling protein (UCP)-2, a phenomenon known to be induced during conditions of increased oxidative stress, resulting in increased QO_2 unrelated to ATP production [11, 21]. Importantly, diabetes-induced mitochondria uncoupling via UCP-2 is prevented by antioxidant treatment [22]. In the present study, addition of H_2O_2 to wildtype cells decreased transport-dependent oxygen consumption and did not affect transport- independent oxygen consumption, arguing against the presence of mitochondria uncoupling in these cells. However, it has been shown that mitochondria uncoupling is regulated by oxidative stress originating from the matrix side of the electron transport chain [23] and addition of H_2O_2 may therefore not be a strong enough signal to increase mitochondria uncoupling in cells with normal levels of oxidative stress. However, in cells with increased oxidative stress an increase in transport- independent oxygen consumption was evident, suggesting increased mitochondria uncoupling. Interestingly, the specific effect of Ang II on increasing transport- independent QO_2 may indeed be due to increased mitochondrial uncoupling. In a study by Doughan et al., addition of Ang II to cultured bovine aortic endothelial cells increased mitochondrial superoxide production and mitochondrial uncoupling [24]. Recently, Ang II receptors have been localized to the mitochondrial inner membrane [25]. In kidneys from SHR rats, oxidative stress was increased and the mitochondria displayed increased H_2O_2 generation, decreased membrane potential and increased UCP-2 expression [26]. Importantly, the effect of Ang II on transport- independent QO_2 was not replicated by increased oxidative stress *per se*, implying a crucial and specific role for AT1-R signaling. It is tempting to speculate that AT1-R located in the mitochondria are involved in these specific effects of Ang II. However, there was no effect of Ang II on transport-independent QO_2 in cells with increased levels of oxidative stress, demonstrating that oxidative stress may still have a regulatory role in mediating the observed effect on transport-independent QO_2 in wild-type cells. Reduced AT1-R expression or blunted receptor response in conditions with increased oxidative stress cannot be excluded.

In conclusion, the present study demonstrates that Ang II can affect both transport-dependent and transport-independent QO_2 in mouse proximal tubular cells and may be an important pathway modulating renal QO_2.

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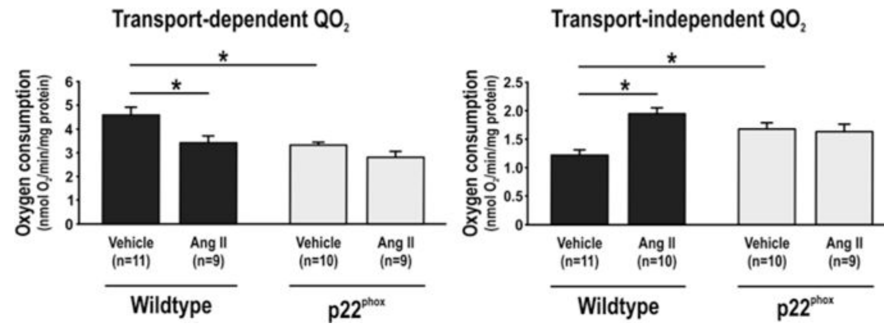

Fig. 21.1.

Transport-dependent (left) and transport-independent oxygen consumption (QO_2, right) in immortalized wild-type mouse proximal tubular cells and corresponding cells overexpressing the NADPH oxidase subunit $p22^{phox}$ and the effect of 48 h exposure to angiotensin II. *Asterisk* denotes $p < 0.05$ and data are presented as mean ± SEM

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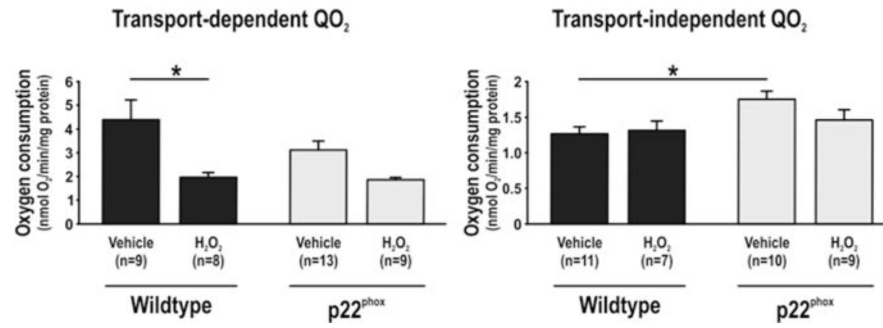

Fig. 21.2.

Transport-dependent (left) and transport-independent oxygen consumption (QO_2, right) in immortalized wild-type mouse proximal tubular cells and corresponding cells overexpressing the NADPH oxidase subunit $p22^{phox}$ and the effect of 48 h exposure to H_2O_2. *Asterisk* denotes $p < 0.05$ and data are presented as mean ± SEM

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