Impact of elevated dietary sodium intake on NAD(P)H oxidase and SOD in the cortex and medulla of the rat kidney

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Submitted 28 August 2009; accepted in final form 22 April 2010

Hypothesis

There is now a clear consensus that, in many cardiovascular diseases, such as hypertension, heart failure, diabetes, and obesity, a state of oxidative stress exists where there is a raised production of reactive oxygen species that has potentially damaging effects on proteins, lipids, and DNA or react with nitric oxide (NO), thereby removing its functional role in physiological control mechanisms (17, 20). Oxidative stress results from an imbalance between the generation of reactive oxygen species, such as superoxide anion (O\textsuperscript{2-}), hydrogen peroxide, and hydroxyl radical, and the antioxidant defense systems, such as superoxide dismutase (SOD) (18).

NAD(P)H oxidases are responsible for the generation of reactive oxygen species, and in unstimulated phagocytes, where it was originally identified, the NAD(P)H oxidase enzyme is composed of gp91\textsuperscript{phox}, a membrane-integrated catalytic subunit associated with p22\textsuperscript{phox}, an accessory subunit. The activator (p67\textsuperscript{phox}) and the organizer/adaptor subunits (p47\textsuperscript{phox} and p40\textsuperscript{phox}) exist in the cytoplasm and, upon activation, translocate to the membrane to assemble the active oxidase that transfers electrons from the substrate to O\textsubscript{2-}, forming O\textsubscript{2}•⁻. Phagocytic gp91\textsuperscript{phox} [NAD(P)H oxidase (NOX) 2] has also been detected in vascular, cardiac, renal, and neural cells while its homolog NOX4 is abundant in the kidney and in vascular smooth muscle cells (6, 13). The activities of the NOX homologs are regulated differently; while NOX1–3 function as multicomponent complexes involving the aforementioned cytosolic regulator proteins, NOX4 exhibits high constitutive activity and is not dependent on any of these regulators (16). O\textsubscript{2}•⁻ is normally present at low levels because of the scavenging activity of SOD, which generates the product hydrogen peroxide, itself a bioactive molecule that is broken down to water by the intracellular enzyme catalase or, under the influence of ferric ions, converted into the potent and damaging hydroxyl radical (OH•). SOD exists in three isoforms that are localized to different subcellular/extracellular compartments: SOD1 (cytoplasm), SOD2 (mitochondria), and SOD3 (extracellular). SOD1 and SOD3 are Cu/Zn SODs while SOD2 is a MnSOD based on metal ion cofactor requirements (18). It is now accepted that a decrease in SOD activity leads to raised O\textsubscript{2}•⁻ levels that can potentially disrupt cellular mechanisms and function.

One of the key organs at the center of cardiovascular homeostasis is the kidney, which comprises both vascular and epithelial cells which are necessary for both hemodynamic and excretory function (8). The renal cortex is where bulk filtration and reabsorption occurs, whereas the medullary area has a low blood flow essential for the maintenance of interstitial concentration gradients. The medulla is an area that is significant in determining the level at which blood pressure may be set (7) and is a relatively hypoxic area and under oxidative stress (1). This is exacerbated in hypertension as demonstrated in recent proteomic studies in the medulla of the spontaneously hypertensive rat where there was enhanced nitration of proteins involved in NO signaling and antioxidant defense (22) as well as raised carbonylation of enolase 1 and catalase (23).

Lenda and Boegehold (14) reported that exposure of rats to a high-salt diet resulted in a raised oxidant activity within the walls of arterioles in the skeletal microvasculature which they attributed to increased NAD(P)H oxidase activity (15). More recently, Kitiyakara et al. (12) found in exposure of rats to an elevated dietary sodium intake enhanced renal cortical g91\textsuperscript{phox} mRNA that was associated with an increased NAD(P)H oxidase activity but decreased SOD mRNA. However, whether this fall in SOD mRNA translated into reduced activity was not evaluated; moreover, whether there were comparable responses in the renal medulla was not investigated.

The aim of the present study was to further explore the impact of a raised dietary sodium intake on NAD(P)H oxidase activity and SOD in the cortex and medulla of the rat kidney.
and SOD activities and on the expression of NAD(P)H oxidase subunits and SOD not only in the renal cortex but also in the renal medulla where the situation is unclear. The hypothesis tested was that placing rats on an elevated dietary sodium intake would result in oxidative stress in both the renal cortex and medulla, causing NAD(P)H oxidase activity to be raised and that of SOD decreased.

**METHODS**

The experimental procedures were performed under the European Committee Directive 86/609/EC and in compliance with the Irish Animal License awarded to E. J. Johns (B100/3260). The protocols were approved by the University College Cork Animal Experimentation Ethics Committee.

**Animals and dietary regimen.** Eight- to nine-week-old male Wistar rats (150–200 g; Harlan, Oxon, UK) were housed in the Biological Services Unit at University College Cork. Two dietary regimes were used, one in which the rats were fed either a normal diet containing 0.3% sodium or high-salt diet containing 3% sodium, for a period of 2 wk. The normal sodium diet was supplied by Harlan Teklad (Bicester, Oxon, UK), and the high-salt diet was supplied by Lillico (Surrey, UK); both had similar carbohydrate, fat, and protein compositions. All rats had access to the diet and tap water ad libitum (Surrey, UK); both had similar carbohydrate, fat, and protein compositions. Both cortical and medullary tissues were homogenized in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) with 100 strokes of a dounce homogenizer. Following incubation on ice for 20 min, homogenates were centrifuged at 10,000 g at 4°C for 10 min. Supernatants were transferred to fresh tubes, and protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA).

**Enzyme assays.** Both cortical and medullary tissues were homogenized in lysis buffer (20 mM KH2PO4, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) with 100 strokes of a dounce homogenizer. The homogenates were centrifuged at 10,000 g at 4°C for 10 min, and the supernatant was removed, frozen in liquid nitrogen, and then transferred to a −80°C freezer until use. Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA).

**Western blots.** Both cortical and medullary tissues were homogenized in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) with 100 strokes of a dounce homogenizer. Following incubation on ice for 20 min, homogenates were centrifuged at 10,000 g at 4°C for 10 min. Supernatants were transferred to fresh tubes, and protein concentrations were determined using a Bradford assay kit (Bio-Rad). Samples were frozen in liquid nitrogen and then stored at −80°C until use.

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**Protein extracts (100 μg) were resolved on both 15% SDS-polyacrylamide gels (anti-SOD Western blotting) and 10% SDS-polyacrylamide gels [anti-NAD(P)H oxidase subunit Western blotting] and were then electrophoretically transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Bucks, UK). Membranes were blocked for 1 h in TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween) containing 5% nonfat dried milk and were incubated overnight with the following dilutions of primary antibody in 5% nonfat dried milk: anti-NOX2, 1:1,000 (Upstate Biotechnology, New York, NY); anti-NOX4, 1:1,000 (Abcam, Cambridge, UK); anti-p73phox (Millipore, Billerica, MA); anti-MnSOD (SOD2), 1:1,000 (BD Biosciences, San Jose, CA); and anti-Cu/Zn SOD (SOD1), 1:1,000 (BD Biosciences). After incubation for 1 h with a 1:4,000 dilution of horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Danvers, MA), bands were visualized using enhanced chemiluminescence (ECL Plus; GE Healthcare). Resultant films were digitally photographed, and densitometric analysis of bands was performed (UviPhotoMW; Solartech, Harrison, MI).

**Statistics.** Data, means ± SE, were analyzed using a one-way ANOVA followed by Tukey’s post hoc test with significance taken at P < 0.05.

**RESULTS**

It can be seen in Fig. 1A that, in the rats maintained on a normal dietary sodium intake, urine flow remained at a stable level over the 2-wk period, but that urine flow in the animals placed on a high-dietary-sodium intake was increased by some 10-fold (P < 0.001) on days 7/8 and remained at this elevated rate on days 14/15. In the animals given a normal sodium diet and the apocynin (Fig. 1A), basal urine flow was no different from that recorded in the group not receiving the drug, and it remained at this level on days 7/8 and 14/15. Administration of the high-sodium diet plus apocynin (Fig. 1A) resulted in a marked elevation in urine flow, by some eightfold at both days 7/8 and 14/15 (both P < 0.001), and the magnitude of these changes was no different from those obtained in the absence of apocynin (Fig. 1A).

Figure 1B presents the fractional sodium excretion in the rats receiving either a normal or high-sodium diet, and it can be seen that, in the rats receiving a normal sodium diet, urine flow was at a constant level over the 15-day period but was in-
creased by some 8- to 10-fold once the animals were placed on the high-sodium diet on days 7/8 and 14/15 (both $P < 0.001$). A similar pattern of change was seen in the rats given apocynin on either the normal or high-salt diet (Fig. 1B) where the animals on the normal diet had a low stable fractional sodium excretion over the 15-day period, whereas, in the rats on a high-sodium diet, there were marked elevations in this variable on days 7/8 and 14/15 (both $P < 0.001$).

The rate of 8-isoprostane excretion in the rats on a normal sodium diet was the same at baseline as at days 7/8 and 14/15 (Fig. 1C), whereas, in the rats on a high-sodium diet, 8-isoprostane excretion rates increased by some 14- to 15-fold on days 7/8 and remained at this elevated rate on days 14/15 (both $P < 0.001$). In the rats given apocynin (Fig. 1C) on a normal sodium diet, isoprostane excretion did not change in any meaningful way over the 15-day period. Administration of the apocynin to the rats given the high-sodium diet (Fig. 1C) resulted in a doubling of their 8-isoprostane excretion rates on days 7/8 and 14/15 (both $P < 0.001$), but the magnitudes of these changes were markedly attenuated compared with the 14-
The activity of the NAD(P)H oxidase is given in Fig. 2, and it can be seen that, in the renal cortex of the rats maintained on a high-salt diet, it was ~100% (P < 0.05) higher than that of the rats on the normal sodium diet. By contrast, NAD(P)H oxidase activity was at similar levels in the medullas of the rats maintained on the normal and the high-sodium diet (Fig. 2). The renal protein expression of the NOX2, NOX4, and p47phox subunits of NAD(P)H oxidase in rats fed both normal and high-salt diets was also investigated. Compared with a normal sodium diet, a high-sodium diet caused an approximately twofold increase (P < 0.05) in the expression of NOX2 and p47phox with no changes in the levels of NOX4 in the renal cortex of the rats (Fig. 3A), as determined by the Western blotting and densitometric analysis. However, it was apparent from both the Western blots and the densitometry measurements that expression of the NOX2, NOX4, and p47phox subunits of NAD(P)H oxidase in the medulla (Fig. 3B) was no different in the animals on either a normal or a high-dietary-sodium intake.

Figure 4 presents the SOD activity in the cortices and medullas of rats maintained on either the normal or high-dietary-sodium diets. Within both groups, activities per milligram protein of both SOD isoforms were greater in medullary tissue compared with cortical tissue. However, dietary sodium intake itself had no significant effect on either Cu/Zn SOD or MnSOD activities.

The effect of high-salt diet on the expression of both Cu/Zn SOD (SOD1) and MnSOD (SOD2) in the cortical and medullary tissues was also investigated by Western blotting and densitometric analysis. It can be seen in Fig. 5, A and B, that the expression levels of SOD1 and SOD2 in both the cortical and medullary tissues were the same in rats maintained on the normal and the high-sodium diets.

**DISCUSSION**

Oxidative stress is recognized as a state where tissues are challenged and tissue metabolic activity is raised. Although there is a general acceptance that this occurs in cardiovascular (25) and renal (4, 20, 21) diseases, it is less clear whether oxidative stress is a response to more physiological situations. The degree of oxidative stress in an organ, that is, the generation of reactive oxygen species, will be a balance between the rate of production, a function of NAD(P)H oxidase (4, 12), and the level of scavenging activity by extracellular and cytosolic SOD (18). Thus, in any situation, it is important to know how the activity of each of these enzymes is changed.

The first step in this investigation was to determine the impact of a high-dietary-sodium intake on oxidative stress in the kidney. To this end, we examined the effect of an elevated dietary sodium intake on the excretion of one of the metabolic indicators of oxidative stress, 8-isoprostane. It was evident that, even after 7 days of the high-sodium intake, there was a marked increase in 8-isoprostane excretion that was maintained at the 14-day time point. In an attempt to provide support that
the increased 8-isoprostane excretion was the result of raised NAD(P)H oxidase activity, a further group of rats was treated with apocynin (19) and subjected to the raised sodium intake. Apocynin has been shown to interfere with the protein complexing of the NAD(P)H oxidase subunits, thereby interfering and decreasing its activity (9, 19). It was clear from our findings that the increase in 8-isoprostane excretion in response to the raised sodium intake was markedly blunted, supporting the view that the high-dietary-sodium intake was indeed raising the level of oxidative stress. Nonetheless, 8-isoprostane excretion rates were still elevated above those of the rats on a normal diet. The reasons for this are unclear but could be a consequence of the dose of apocynin used or because of reactive oxygen species generation by another pathway. Interestingly, the ability of the kidney to excrete the increased sodium as a result of elevated dietary intake was not impaired when the apocynin was given. These findings are consistent with those reported earlier (12) that raising, but not lowering, dietary sodium intake constitutes a metabolic challenge and increases O2 generation in renal homogenates. One limitation from this approach was that it was not possible to indicate the source of the 8-isoprostane metabolite that is excreted, whether it arises from within the interstitium of the kidney itself or whether it is filtered from the blood. It is likely that both sources are contributing.

The subsequent step was to evaluate the effect of high-salt intake on renal NAD(P)H oxidase activity using a lucigenin assay, and on renal levels of two homologues of the enzyme complex catalytic subunit (NOX2 and NOX4) and a key regulatory subunit, p47phox, by Western blotting analysis. We specifically examined whether any changes in enzyme activity levels were the same or different in the two major regions of the kidney, the cortex and medulla. It was quite evident that, within the cortex, NAD(P)H oxidase activity was significantly elevated in the rats placed on the high-sodium diet for 14 days. The elevated activity may be partially explained by the observation that the high-salt intake also increased expression of both NOX2 and p47phox in the cortex. Interestingly, the high-sodium diet had no effect on the cortical expression of NOX4. In the medullary tissue, NAD(P)H oxidase activity levels were comparable to those of the cortex in the animals on the regular sodium diet; the levels were no different in the animals provided with the high-sodium diet. This finding was reinforced by the Western blotting data which showed that levels of NOX2, NOX4, and p47phox in the medulla of the animals exposed to the high-salt were no different from that in the animals fed a regular diet.

These findings are in support of the report by Kitiyakara et al. (12) who demonstrated that, in renal cortical tissue, the mRNA expression of NOX2 and p47phox but not NOX4 was significantly increased by raising sodium intake. Our findings extend these earlier observations and show that these changes in NAD(P)H oxidase activity and expression do not occur in the medulla. Taken together, these data demonstrate it is at the cortex, where the majority of salt and water handling occurs, that oxidative stress levels are increased. By contrast, within the medulla, the area at which discrete adjustments in fluid output are made and possibly requiring smaller demands in

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**Fig. 5.** SOD1 (Cu/Zn SOD), SOD2 (MnSOD), and actin protein expression in the cortex (A) and medulla (B) in rats exposed to a normal sodium and high-sodium diets. SOD1 and SOD2 band intensities were divided by corresponding actin band intensities, and results are presented as relative band intensities (n = 3).
metabolic energy, there was no measurable change in the activity levels of the enzyme generating reactive oxygen species. This would mean that the actions of $O_2^-$, either directly or indirectly, to influence vascular tone in the medulla would be unchanged. Thus, since medullary blood flow is crucial in determining blood pressure (7), an unchanged blood flow in this region might underlie, in part, the observations (15, 26) that blood pressure is unchanged when dietary sodium is elevated.

Although originally identified in phagocytic white blood cells, the catalytic subunit gp91phox (NOX2) is also expressed in renal tissues (5). Recent research has shown that NOX2 is the primary source of $O_2^-$ at the macula densa induced by NaCl where it plays an important role in the enhancement of tubuloglomerular feedback by scavenging NO, resulting in a vasoconstriction of afferent renal arterioles (27). Furthermore, NOX2 plays an important role in the renal microcirculatory responsiveness to ANG II and adenosine and may contribute to ANG II-induced hypertension (2). Our finding that high-salt intake specifically increases NOX2 expression in the cortex is consistent with its involvement in the generation of renal $O_2^-$ particularly at the macula densa. NOX4 was originally cloned from the kidney and is abundantly expressed in the renal cortex and the vasculature (10). For this reason, we also examined its expression following high-salt intake. Unlike NOX2, there were no changes in NOX4 expression in either the cortex or medulla in rats fed a high-sodium diet. Most studies have indicated that the NOX4 catalytic subunit generates a significant amount of superoxide in a constitutive manner without the requirement of other membrane or cytosolic subunits. It is generally considered that, while it contributes to significant basal $O_2^-$ production in the kidney, its expression and activity are not as tightly regulated as NOX2 (16). The lack of an effect of salt on NOX4 demonstrated in our in vivo study (protein abundance) and that of Kitiyakara et al. (12) (mRNA abundance) is supported by a recent in vitro study that showed that high salt did not increase NOX4 activity in macula densa cells (27). p47phox phosphorylation is a key event that triggers the association of the ternary cytoplasmic complex of p67phox, p47phox, and p40phox with the inactive membrane-integrated NOX/p22phox subunits creating active NAD(P)H oxidase (16). Our finding that p47phox protein expression was upregulated in the cortex of high-salt-fed rats builds on the findings of Kitiyakara et al. (12) that raised dietary salt increased p47phox mRNA abundance.

An elevation of NAD(P)H oxidase activity will result in increased generation of $O_2^-$ having the potential of causing tissue damage. However, this will be dependent on whether the scavenging abilities of the SODs are raised to a similar degree; if they are, the oxidative stress levels will be essentially unaltered. This was explored in the current study by determining the impact of the high-dietary-sodium intake on Cu/Zn and MnSOD activity and on the expression of SOD1 (cytoplasm) and SOD2 (mitochondria) isoforms in the cortex and medulla of the kidneys. It has been shown previously that the activity of extracellular space SOD (SOD3) is very low in rat tissues, and it is well accepted that SOD1 and SOD2 activities contribute predominantly to the total activity of SOD in rats (3). The findings were quite clear in that placing the rats on the elevated dietary sodium intake had no effect on either the levels of SOD1 and SOD2 protein expression or Cu/Zn and MnSOD enzyme activity in the cortex and the medulla. These observations are somewhat different from those reported by Kitiyakara et al. (12) where cortical SOD1 and SOD2 (but not SOD3) mRNA abundances decreased as dietary sodium intake was increased. The reasons for this disparity with our data are unclear but may reside in the somewhat different experimental scenarios in the two studies where Sprague-Dawley rats were used and the diet contained a higher sodium content (6% sodium) but with a shorter exposure duration (7 days). It should also be pointed out that no SOD activity assays were performed in the earlier study so it is not certain if reductions in mRNA levels correlate with reductions in dismutase activity. Although the 3% sodium diet in our study did stimulate increased NAD(P)H oxidase expression/activity, perhaps a diet with a higher sodium concentration would have also caused reduced SOD expression/activity with increased generation of reactive oxygen species.

The present study provided novel insight as to the situation in the medulla. It was evident that the level of SOD activity was higher in the medulla compared with that in the cortex. This raised medullary level of SOD activity may reflect the fact that this region is relatively hypoxic (1) and therefore is at a raised basal level of oxidative stress, even under normal conditions. Importantly, it was evident that, as with the cortex, placing the animals on the high-sodium diet for 14 days did not change the activity or protein expression of either isofrom of SOD. This observation is particularly significant in that it means that the balance between generation of $O_2^-$ and the degree to which they are scavenged is unaltered when dietary sodium intake is elevated. Thus it is unlikely that medullary blood flow will be altered on exposure to a high-dietary-sodium intake via $O_2^-$ as their production and rate of degradation will be unchanged. This may, in part, be a reason why blood pressure in normal rats changes only minimally when challenged with a short period of raised dietary sodium (15, 26). However, medullary blood flow will be subject to the influence of a number of other local and circulating factors that may determine the level at which it is set.

Perspectives and Significance

This study set out to examine the impact of high-dietary-sodium intake on the oxidative challenge posed to the kidney. Two weeks of a high-sodium intake resulted in an elevated sodium excretion associated with a raised output of 8-isoprostane, a metabolic marker of oxidative stress, and this contention was supported by the finding that it was suppressed by the NAD(P)H blocker apocynin. Within the kidney, cortical NOX2 and p47phox protein expression and overall NAD(P)H oxidase activity was raised by the exposure to the high dietary intake of sodium, but this was at a time when the expression and activity of the SOD informes were unchanged. These findings suggested that raised generation of reactive oxygen species in the cortex as a consequence of the high-sodium diet was not compensated for by increased scavenging by SOD. It is this region of the kidney that has to ensure that bulk fluid reabsorptive rates are adjusted to ensure that the raised dietary sodium intake can be excreted. By contrast, NAD(P)H oxidase and SOD activities and NOX2, p47phox, SOD1, and SOD2 expression in the medulla were unaltered by increasing the sodium content of the diet. Blood flow through the medulla has been identified as a
key factor in determining the level of blood pressure. The findings that the production and degradation of O2 are not closely related to the level of blood pressure. The production of O2 is altered by elevation of sodium intake. This may explain, in part, why blood pressure is not normally altered by elevation of sodium intake.

DISCLOSURES

No conflicts of interest are declared by the authors.

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