Effect of apocynin treatment on renal expression of COX-2, NOS1, and renin in Wistar-Kyoto and spontaneously hypertensive rats


Effect of apocynin treatment on renal expression of COX-2, NOS1, and renin in Wistar-Kyoto and spontaneously hypertensive rats. Am J Physiol Regul Integr Comp Physiol 290: R694–R700, 2006; doi:10.1152/ajpregu.00219.2005.—Macula densa (MD) cells of the juxtaglomerular apparatus (JGA) synthesize type 1 nitric oxide synthase (NOS1) and type 2 cyclooxygenase (COX-2). Both nitric oxide (NO) and prostaglandins have been considered to mediate or modulate the control of renin secretion. Reactive oxygen species (ROS) produced locally by NADPH oxidase may influence NO bioavailability. We have tested the hypothesis that in hypertension elevated ROS levels may modify the expression of NOS1 and COX-2 in the JGA, thereby interacting with juxtaglomerular signaling. To this end, spontaneously hypertensive rats (SHR) and Wistar-Kyoto control rats (WKY) received the specific NADPH oxidase inhibitor, apocynin, during 3 wk. Renal functional and histochemical parameters, plasma renin activity (PRA), and as a measure of ROS activity, urinary isoprostane excretion (IP) were evaluated. Compared with WKY, IP levels in untreated SHR were 2.2-fold increased, and NOS1 immunoreactivity (IR) of JGA 1.5-fold increased, whereas COX-2 IR was reduced to 35%, renin IR to 51%, and PRA to 7%. Apocynin treatment reduced IP levels in SHR to 52%, NOS1 IR to 69%, and renin IR to 62% of untreated SHR, whereas renin mRNA, COX-2 IR, glomerular filtration rate, PRA, and systolic blood pressure remained unchanged. WKY revealed no changes under apocynin treatment. These data show that NADPH oxidase is an important contributor to elevated levels of ROS in hypertension. Upregulation of MD NOS1 in SHR may have the potential of blunting the functional impact of ROS at the level of bioavailable NO. Downregulated COX-2 and renin levels in SHR are apparently unrelated to oxidative stress, since apocynin treatment had no effect on these parameters.

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and COX-2 and that this could exert secondary effects on renin synthesis and secretion.

The present experiments were performed to assess whether the formation of ROS by local NADPH oxidase may be a factor that could modify the expression of NOS1 and COX-2 in the JGA. To reduce the local formation of ROS, the specific NADPH inhibitor apocynin was systemically administered to Wistar-Kyoto (WKY) rats and SHR. Apocynin binds to essential thiol groups of NADPH oxidase subunits and thus prevents the transfer of the soluble cytosolic subunits p47phox and p67phox to the membrane, an event that is essential for the assembly of the functional NADPH oxidase complex (11). Our data show that apocynin treatment had no effect on the expression of JGA enzymes or plasma renin in control rats. On the other hand, in SHR apocynin significantly reduced the elevated NOS1 expression without changing the levels of COX-2 or renin mRNA, whereas renin immunoreactivity was suppressed. Thus it appears that the increased formation of ROS in SHR stimulates the expression of the NOS1 enzyme, an adjustment that could serve to compensate for the enhanced degradation of NO.

MATERIAL AND METHODS

Animals and tissue preservation. SHR and control WKY rats were obtained from Charles River Laboratories at the age of 7 wk. Animals were housed in the National Institutes of Health (NIH) animal facility and divided into groups of five. One group of WKY and SHR had free access to standard laboratory diet and tap water, whereas another received a 2.5 mM solution of apocynin (Sigma) dissolved in tap water. After 3 wk of treatment, rats were placed in metabolic cages to assess water intake and to collect a 24-h urine sample. Blood pressure was measured by tail cuff plethysmography in conscious animals; to minimize stress during the measuring procedure, rats were accustomed to the holding device for 2 h on three consecutive days. At least 30 individual measurements were performed and averaged at the end of the third day. Quality of the measurements was ensured by monitoring the pulse amplitude curve and discarding measurements influenced by artifacts. For perfusion fixation, animals were anesthetized, the abdominal cavity was opened, and a blood sample was taken from the inferior vena cava. A catheter was then placed in the aortal aorta, and the kidneys were flushed for 45 s with sucrose/PBS (pH 7.35, 330 mosmol/kg H2O) at a flow rate of 0.66 ml/s. Subsequently, the tissue was perfused for 5 min with 3% paraformaldehyde (PFA) in PBS (pH 7.35) at a flow rate 0.4 ml/s. After fixation, kidneys were removed and processed for further procedures. A 3-mm coronal section from the center of the left kidney was postfixed in 3% PFA for 12 h and embedded in paraffin. The remaining parts of the left kidney were cryoprotected in sucrose/PBS (pH 7.35, 800 mosmol/kg H2O) for 12 h and shock-frozen in liquid nitrogen-cooled isopentane.

Immunohistochemistry. Cryostat sections (5 µm thick) were cut for NOS1, COX-2, and renin immunocytochemistry. Sections were incubated in 0.5% Triton X-100/PBS for 30 min. After being rinsed in PBS, unspecific protein-binding sites were blocked by incubation with 5% dry milk in PBS. Primary antibodies were applied in 5% dry milk in PBS. For NOS1 immunostaining, a polyclonal antibody directed against amino acids 237–250 of a synthetic peptide corresponding to amino acids 237–250 of the endothelial cell-specific NOS1, COX-2, and renin immunostaining. Sections were incubated in 0.5% Triton X-100/PBS for 30 min. After being rinsed in PBS, bound antibody was detected with a peroxidase-labeled secondary antibody (rabbit anti-mouse renin; gift of T. Inagami, Nashville, TN) at a 1:10,000 dilution. Bound antibody was detected with a peroxidase labeling kit (Vector ABC). Immunoreactive signals for NOS1 and COX-2 were quantified by counting stained macula densa cells and normalizing for the total number of glomeruli; similarly, renin immunoreactivity was quantified by counting immunoreactive JGA (32).

Renin mRNA in situ hybridization. Renin mRNA expression was evaluated using in situ hybridization, as previously described (8). Briefly, a riboprobe was generated by in vitro transcription from a specific renin cDNA template (1) using digoxigenin (DIG)-labeled UTP (Roche). T3 and T7 RNA polymerases were used to obtain sense and antisense probes. In situ hybridization was performed using 1-µm coronal sections mounted on silane-coated slides. Sections were postfixed by immersion in ice-cold 4% PFA in PBS and acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride followed by a dehydration process in a graded ethanol series. Sections were hybridized with 5–10 ng/ml probe at 47°C for 18 h and rinsed in decreasing concentrations of sodium citrate and buffer 1, which contained Tris·HCl (1 M) and NaCl (0.15 M) at a pH of 7.5. After blocking unspecific protein-binding sites by a 30-min incubation with blocking medium (1%) and BSA (0.5%) in buffer 1 (Roche) bound RNA was detected using an alkaline phosphatase-labeled sheep anti-DIG Fab fragment diluted 1:500 in blocking medium. Signal was generated with 4-nitro blue tetrazolium (45 µl), 5-bromo-4-chloro-3-indolylphosphate (35 µl), and 2.5 mg levamisole in 10 ml of a buffer containing Tris·HCl (0.1 M), NaCl (0.1 M), and MgCl2 (0.05 M) at a pH of 9.5. The progress of color development was monitored using standard bright-field microscopy. The reaction was stopped when the signal was clearly distinguishable and background staining did not yet occur. Slides were then rinsed and covered with a cover slip in PBS-glycerol.

Laboratory parameters. Plasma renin activity was determined by RIA of generated ANG I as previously described (17). Urinary 8-isoprostane concentration was measured by enzyme-linked immunosay using the manufacturer’s protocol (catalog no. 516351; Cayman Chemicals). Plasma and urinary creatinine was measured in the clinical chemistry facility of the NIH. Creatinine clearance was determined using the standard formula.

Statistical analysis. All values are given as means ± SE of 4–5 animals in each group. Statistical analysis was performed using a two-tailed Student’s t-test. Null hypothesis has been excluded when P was < 0.05.

RESULTS

Blood pressure and urinary isoprostane levels. At the end of the treatment period, systolic blood pressure was 176 ± 3 mmHg in control SHR compared with 132 ± 6 mmHg in WKY (P < 0.005). Apocynin treatment had no significant effect on blood pressure in either strain (Fig. 1). Baseline urinary isoprostane excretion was higher in SHR compared with WKY (32.4 ± 3.8 vs. 14.8 ± 3.3 ng/dl; P < 0.01). Apocynin treatment lowered isoprostane levels significantly in SHR (18.7 ± 4.6 ng/dl; P < 0.05), whereas no change was found in WKY (Fig. 2). Creatinine clearance was determined in SHR [1.23 ± 0.15 in the untreated vs. 0.99 ± 0.13 ml/min in the treated group; not significant (NS)].

NOS1 expression. The number of NOS1 immunoreactive cells was 50% higher in SHR compared with WKY at steady state (179 ± 11 vs. 119 ± 11 positive cells/100 glomeruli;
P < 0.005). Apocynin treatment significantly lowered the number of NOS1-expressing cells in SHR (125 ± 9; P < 0.005), whereas it had no effect in WKY [105 ± 16 (NS); Fig. 4].

**COX-2 expression.** The number of COX-2 immunoreactive cells in terminal TAL and macula densa was lower in SHR than in WKY at steady state (23.5 ± 3 vs. 66 ± 7.2 positive cells/100 glomeruli; P < 0.05). Apocynin treatment had no significant effect in either SHR or WKY [19 ± 4 in SHR (NS) and 70 ± 8 in WKY (NS); Fig. 5].

**Renin levels.** The fraction of JGA showing a renin mRNA signal by in situ hybridization was lower in untreated SHR compared with untreated WKY (10 ± 3 vs. 23 ± 4 positive JGA/100 glomeruli; P < 0.01). Apocynin treatment had no effect on renin mRNA expression in either SHR [7.2 ± 1.2 (NS)] nor in WKY [26 ± 1.6 (NS); Fig. 6]. Renin immunoreactivity was lower in control SHR than in WKY as well (16 ± 1.4 vs. 31 ± 2.4 positive JGA/100 glomeruli; P < 0.04). Administration of apocynin caused a significant reduction in the renin immunoreactive signal in SHR (9.9 ± 0.6, P < 0.01), whereas no change was seen in WKY [30 ± 0.85 (NS); Fig. 7]. Plasma renin activity was lower in SHR than in WKY at steady state (0.43 ± 0.17 vs. 6.0 ± 0.75 ng ANG I·ml⁻¹·h⁻¹; P < 0.01) and remained unaltered after apocynin treatment in both SHR [0.23 ± 0.1 (NS)] and WKY [9.7 ± 2.31 (NS); Fig. 8].

**DISCUSSION**

The focus of the present study was to investigate the possibility that ROS may be involved in the regulation of NOS1 and COX-2, two enzymes believed to be important for the regulatory pathways that connect macula densa and afferent smooth muscle cells. Specifically, by altering the expression of COX-2 and NOS1, it is conceivable that ROS secondarily affect synthesis or secretion of renin. Our results show that chronic and systemic application of apocynin, a potent inhibitor of the superoxide-generating NADPH oxidase, led to a reduction of NOS1 immunoreactivity of macula densa cells in SHR, whereas NOS1 levels were not affected by apocynin in WKY rats. Because basal expression of NOS1 in untreated SHR is constitutively elevated compared with WKY, the effect of inhibition of NADPH oxidase consisted of a normalization of NOS1 levels in SHR. Steady-state COX-2, renin mRNA, and plasma renin activity levels were lower in SHR than in WKY and were not markedly changed by apocynin. Neither blood pressure nor creatinine clearance was altered by apocynin in either WKY or SHR. The fraction of JGA displaying renin immunoreactivity was significantly lower in the treated SHR compared with the untreated SHR. The urinary excretion of isoprostanes was used as an index of overall renal superoxide production. 8-Isoprostane F₂α is the major urinary metabolite of F₂-isoprostanes and is formed by peroxidation of arachidonic acid independent of COX enzyme pathways. Isoprostane formation has been successfully used as a biomarker for states of oxidative stress in a variety of animal and human investigations (18). Our results confirm that SHR have an increased excretion of isoprostanes compared with WKY, supporting the existence of oxidative stress in these animals (23). Direct measurements of superoxide generation have also shown a three- to fivefold elevation in stroke-prone SHR (15). The 43% reduction of urinary 8-isoprostane excretion produced by apocynin in SHR indicates that NADPH oxidase is at least in part responsible for the increase in superoxide formation. It is noteworthy that the effect of apocynin on isoprostane excretion was similar to that observed in a previous study in which the membrane-permeable superoxide dismutase mimetic tempol was used to reduce superoxide levels (23). In fact, renal NADPH oxidase activity has been shown earlier to be upregulated in SHR (10).

The demonstration of elevated levels of NOS1 in macula densa cells of SHR kidneys is in agreement with similar previous data (33). Furthermore, it agrees with findings that point to a general enhancement of the expression of NOS isoforms in SHR (31). In fact, it has been shown that an increase in endothelial NOS3 in stroke-prone SHR may be directly responsible for enhanced superoxide formation (15). The cytotoxic approach used in our study has the advantage that it permits cell-specific evaluation of NOS1 in macula densa and adjacent TAL cells selectively. Detection of site- and isoform-specific changes in our study may explain why in another study the antioxidant lazoroid (31) caused changes of NOS2 or NOS3 expression, but not NOS1 in whole kidney
homogenates of SHR. It is possible that an alteration of NOS1 production in the small number of macula densa cells was not detectable with this approach (2).

Normalization of juxtaglomerular NOS1 by apocynin indicates that an increased ROS generation by local NADPH oxidase is involved in the upregulation of macula densa NOS1. Exactly how ROS affect the expression of NOS1 in macula densa cells is not entirely clear. However, in view of the well-established role of ROS as activators of a number of transcription factors, it would appear likely that ROS increased

Fig. 3. Quantification of juxtaglomerular parameters. Representative micrographs showing histochemical signal in WKY rats (a, d, g, and j), untreated SHR (b, e, h, and k), and apocynin-treated SHR (c, f, i, and l). a-c: Cy2-labeled nitric oxide synthase (NOS)1 immunoreactivity in WKY rats showing strong expression confined to the cells of the macula densa itself (a), significantly enhanced signal in macula densa (between arrows) and portions of thick ascending limb (TAL) epithelium in the untreated SHR (b), and a reduced number of NOS1 immunoreactive cells in the apocynin-treated SHR (c). NOS1 immunoreactive portions of the macula densa are marked by arrows. d-f: Cy2-labeled cyclooxygenase (COX)-2 immunofluorescence showing strong immunoreactivity in the cells of the macula densa and adjacent cells of the TAL in WKY rats (d), a greatly reduced number of COX-2 immunoreactive cells in the untreated SHR (e), and a nonsignificantly reduced number of immunoreactive cells in the apocynin-treated animals (f). COX-2 immunoreactive macula densa cells are marked by arrows. g-i: Renin immunoperoxidase staining showing normal expression profiles in the WKY rats (g), a reduced number of immunoreactive juxtaglomerular apparatus (JGA) in the untreated SHR (h), and a further significant reduction in the number of immunoreactive JGA in the apocynin-treated SHR (i). Immunoreactive parts of the afferent arteriole are marked by arrows. j-l: Renin in situ hybridization (ISH) showing strong signal in afferent arterioles of WKY rats (j), a reduced number of mRNA positive JGA in the untreated SHR (k), and a nonsignificantly reduced number of renin-expressing JGA in the apocynin-treated SHR (l). Stained cells of the afferent arteriole are marked by arrows.

Fig. 4. Quantification of JGA NOS1 immunoreactivity. Control and apocynin-treated SHR (filled bars) and WKY rats (open bars) are shown. Values (means ± SE) are from at least 100 glomeruli/animal with 4–5 animals/group. \( P < 0.05 \) vs. WKY rats (*) and vs. untreated SHR (#).

Fig. 5. Quantification of JGA COX-2 immunoreactivity. Control and apocynin-treated SHR (filled bars) and WKY rats (open bars) are shown. Values (means ± SE) are from at least 100 glomeruli/animal with 4–5 animals/group. \( *P < 0.05 \) vs. WKY rats.
mRNA expression of NOS1 in the JGA. Conversely, the reduction of NOS1 by apocynin may be the consequence of inhibition of local juxtaglomerular NADPH oxidase. Previous studies have shown that tempol restores the NO-dependent blunting of TGF and that it reverses the general renal vasoconstriction in SHR (23, 33, 37). These observations suggest that the actual NO bioavailability may be augmented by antioxidants despite the reduction in NOS1 expression. The functional importance of this increase in NOS1 expression in untreated SHR may be to counteract to some extent the scavenging of NO that is another consequence of increased ROS formation.

Spontaneous hypertension in rats is usually referred to as a low-renin animal model of hypertension (25), a notion that is confirmed by the present results. Furthermore, the absence of an effect of apocynin indicates that this decrease in renin does not appear to be the direct or indirect consequence of oxidative stress. Although previous studies have reported an increased global renal activity of COX in SHR (16), the specific juxtaglomerular COX-2 expression in SHR in our hands was clearly lower than in WKY. Apocynin treatment did not affect this interstrain difference. Because renin mRNA levels were reduced as well, these observations are in line with previous results that indicated coordinate changes of renin and COX-2 under experimental conditions (9, 32). Suppressed COX-2 levels in the untreated SHR may be related with low NO bioavailability consequent to the effects of ROS. However, because apocynin treatment did not alter COX-2, or the functionally associated expression of renin mRNA, it would appear that NO availability in SHR and in this setting may not have reached control WKY levels.

The dissociation between renin expression and secretion on the one hand, and renal renin content as reflected by the fraction of renin immunoreactive JGA on the other, is intriguing. However, renin biosynthesis follows comparatively complex pathways and includes local and systemic control at different levels, among them NO. NO may facilitate renin production at the transcriptional level, and eventually also modulates its release (5, 8), but effects may be divergent with respect to time frame and quantity requirement of NO. One may therefore speculate that the treatment may have induced this dissociation, possibly by creating altered balance between renin biosynthesis and intracellularly stored renin levels. Of course we also cannot exclude the possibility of a statistical error because of the somewhat limited sample sizes. However, in our hands, quantitation of renin mRNA in situ hybridization and renin immunostaining has provided reproducible results in a number of previous studies with similar sample size (1, 32).

In several previous studies, it has been noted that various antioxidants are able to reduce blood pressure in SHR (20, 22, 37). In contrast, the administration of apocynin in the present experiments was not accompanied by measurable changes in blood pressure in either SHR or control animals. This difference does not seem to be related to differences in the antioxidant efficacy since the reduction in isoprostane excretion was similar to that reported by Schnackenberg and Wilcox (23). We assume that the failure of apocynin treatment to reduce blood pressure in SHR may be related to differences in its effects compared with other antioxidants (35). One such difference may be that antioxidants such as tempol cause central sympathoinhibition to an extent that is not shared by apocynin. Because the hypertension of SHR appears to be at least in part neurogenic, inhibition of sympathetic nerve activity is expected to have hypotensive effects. The exact mechanism of tempol-induced sympathoinhibition is not clear but may involve an altered balance between NO and ROS in the sympathetic nuclei of the rostral ventrolateral medulla where ROS act...
as excitatory mediators of sympathetic activity (36). In another study in which a lazaroid compound was applied in SHR and in which a decrease of blood pressure was observed, the same considerations may be valid (31). In contrast to the present studies in SHR, a significant amelioration of hypertension and renal inflammation has been reported after 3 wk of administration of apocynin in DOCA/salt-treated rats (6). However, in contrast to this model, our rats were studied at an age range (7–10 wk) when blood pressure was still rising, and elevated sympathetic activity and increased vascular contractility primarily determine hypertension in SHR (14, 19, 24). Secondary changes such as vascular and hormonal alterations typically occurring in older SHR and in the DOCA/salt model had not developed at this time point (4, 6, 13). Apocynin treatment in DOCA/salt rats may therefore have contributed to an amelioration of inflammatory parameters that indirectly may aggravate hypertension. Thus we suggest that the principal targets of NADPH oxidase blockade by apocynin may be noninflammatory cells of the renal interstitium, vasculature, and macula densa (3, 10) and that a reduction in ROS formation in these cells has little effect on blood pressure of SHR if inhibition of sympathetic activity does not occur at the same time. Further experiments are needed to investigate this possibility.

In conclusion, our results show that NADPH oxidase is an important contributor to elevated levels of ROS in SHR. However, in contrast to a nonspecific scavenging of ROS by antioxidants or SOD mimetics, a specific blockade of NADPH oxidase does not reduce blood pressure in these animals. Upregulation of macula densa NOS1 in SHR may have the potential of blunting the functional impact of ROS on NO levels. The state of oxidative stress in SHR does not appear to be responsible for the downregulation of renin and COX-2, since the expression of both enzymes was not affected by apocynin.

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