Apocynin but Not L-Arginine Prevents and Reverses Dexamethasone-Induced Hypertension in the Rat

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Background: Dexamethasone (Dex)–hypertension in rats is associated with increased oxidative stress. We investigated effects of the NAD(P)H oxidase inhibitor apocynin and the nitric oxide (NO) precursor L-arginine on Dex-hypertension to determine the relative roles of NAD(P)H oxidase and uncoupling in the reactive oxygen species (ROS) generation and hypertension.

Methods: Male Sprague-Dawley rats (n = 10/group) received Dex (20 µg/kg/day subcutaneously) or saline (vehicle) for 14 days. In a prevention study, rats received 4 days of apocynin treatment (1.5 mmol/L in drinking water) followed by Dex/saline for 12 days. In reversal studies, apocynin or L-arginine was given from day 8 to 14. Systolic blood pressure (SBP) was measured by tail cuff, and thymus weight was used as a marker of glucocorticoid activity.

Results: Administration of Dex increased SBP (104 ± 3 to 122 ± 3 mm Hg, P < .01, mean ± SEM) and decreased thymus and body weight (P’ < .05). Apocynin alone had no effect on SBP, BW, or thymus weight. Apocynin prevented (122 ± 4 Dex, 111 ± 3 mm Hg Apocynin + Dex, P’ < .05) and reversed Dex-hypertension (130 ± 4 to 116 ± 4 mm Hg, P < .01). L-arginine did not reverse Dex-hypertension.

Conclusions: In male SD rats, apocynin but not L-arginine prevented and reversed Dex-hypertension, suggesting that NAD(P)H oxidase–mediated superoxide production but not endothelial nitric oxide synthase uncoupling is important in Dex-hypertension.

Key Words: Apocynin, dexamethasone, hypertension, L-arginine, NAD(P)H oxidase.

Oxidative stress plays a significant role in cardiovascular diseases such as hypertension, hyperlipidemia, diabetes mellitus, ischemic heart disease, and chronic heart failure.1 Oxidative stress occurs when high levels of reactive oxygen species (ROS) overwhelm the endogenous antioxidant system.2 Superoxide, one of the most important ROS,3 is generated through the univalent reduction of oxygen and in turn reacts with other enzymes, producing a series of other ROS.2 Superoxide reacts with nitric oxide (NO), a potent vasodilator,2 to reduce its bioavailability. Superoxide overproduction is common in hypertensive rats. Aortic superoxide production, as detected by lucigenin, was significantly higher than control values in spontaneously hypertensive rats;4 deoxycorticosterone acetate (DOCA)–salt hypertensive rats;5 and angiotensin II–induced hypertensive rats.6

The substance NAD(P)H oxidase is composed of several different subunits (gp91phox [or nox1/nox4], p40phox, p22phox, p47phox, p67phox, and rac), and its components vary in different cell types.7 NAD(P)H oxidase catalyses NAD(P)H to NAD(P)8, releasing a molecule of superoxide.7 Apocynin, a specific NAD(P)H oxidase inhibitor, decreased p22phox mRNA levels in aortic segments from aldosterone-salt male Sprague-Dawley (SD) rats and impeded p47phox subunit assembly within the membrane complex in human endothelial cells to inhibit the activity of NAD(P)H oxidase and its production of superoxide.8 Apocynin inhibited the increase in superoxide production in DOCA-salt hypertensive rats,5 and prevented BP elevation and inhibited NAD(P)H activity and p22phox mRNA levels in rat aortic segments in aldosterone-infused male SD rats.8

Received August 2, 2005. First decision August 31, 2005. Accepted September 20, 2005.

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Published by Elsevier Inc.

AJH 2006; 19:413–418
0895-7061/06/$32.00 © 2006 by the American Journal of Hypertension, Ltd.
Dexamethasone (Dex) is a synthetic glucocorticoid that is commonly used in clinical practice and that increases blood pressure in rats \(^9\) and in human beings. \(^10\) Chronic Dex-treatment increased oxidative stress (as measured by plasma F\(_2\)-isoprostane concentrations) and systolic blood pressure (SBP) in rats, \(^11\) and ROS production in human umbilical vein endothelial cells (HUVEC). \(^12\) Diphenyl-eneiodinium chloride, a NAD(P)H oxidase inhibitor, suppressed the Dex-stimulated increase in ROS production in HUVEC. \(^12\) Tempol, a superoxide scavenger, prevented and partially reversed Dex-hypertension, \(^11\) implying that superoxide overproduction plays a role in Dex-hypertension. However, the source of this superoxide overproduction in Dex-hypertension is unknown.

Dex-hypertension was found to be accompanied by a decrease in serum reactive nitrogen intermediate (NO\(_x\)) concentrations and endothelial nitric oxide synthase (eNOS) mRNA levels in the heart, kidney, and liver in mice. \(^13\) However, \(L\)-arginine did not prevent Dex-hypertension despite increasing the decreased plasma NO\(_x\). \(^14\) Whether \(L\)-arginine supplementation modifies established Dex-hypertension is unknown.

The aims of present study was to investigate the role of NAD(P)H oxidase or \(L\)-arginine supplementation in Dex-hypertension in the rat. The hypotheses of the present study were 1) that apocynin would reverse and prevent Dex-hypertension, and 2) that \(L\)-arginine would not modify Dex-hypertension.

Methods
Animals
This study was approved by the Animal Experimental Ethics Committee of the Australian National University (AEeec Protocol No J.HB. 14.02, 17.03). Male SD rats (Animal Resources Centre, Perth, WA), weighing 200 g, were housed in cages at a constant temperature of 20\(^\circ\)C to 22\(^\circ\)C, humidity 30\% to 31\%, and 12-h light–dark cycle.

Rats had free access to food (standard commercial rodent chow (Gordon’s Specialty Stock Feeds, Yanderra, Australia) and water. Single batches of food were used for each study. Rats were allowed 1 to 2 weeks to acclimatize to their surroundings, handling, and SBP measuring equipment before the experimental procedures.

Protocol
Dexamethasone (20 \(\mu\)g/kg/day, in a volume of 1 mL/kg) (David Bull Laboratories, Mulgrave, Victoria, Australia) or saline (1 mL/kg/day) was administered subcutaneously at 11 AM to 12 noon every day. Apocynin (1.5 mmol/L drinking water) (Fluka, Buchs, Switzerland) and \(L\)-arginine (0.6\% diet) (Sigma-Aldrich, St. Louis, MO) were prepared daily. Water consumption and food intake were measured daily. After 4 control days, rats were randomly divided into seven groups (\(n = 10\) per treatment group).

Experiment 1: Apocynin

Controls Control groups were as follows. Group 1 (Saline treatment): Rats were treated with saline (1 mL/kg/day subcutaneously) and given tap water to drink for 14 treatment (T) days (T0 to T13). Group 2 (Dex treatment): Rats were treated with Dex (20 \(\mu\)g/kg/day subcutaneously) and tap water for 14 days (T0 to T13).

Prevention Study In the prevention study, groups were as follows. Group 3 (Saline+apocynin treatment): Rats were pre-treated (P) with apocynin for 4 days (P0 to P3) followed by saline and apocynin (1.5mmol/L in drinking water) for 12 days (T0 to T11). Group 4 (Dex+apocynin treatment): Rats were pre-treated with apocynin for 4 days (P0 to P3) followed by Dex and apocynin for 12 days (T0 to T11).

Reversal Study In the reversal study, groups were as follows. Group 5 (Saline+apocynin treatment): Rats were treated for 14 days with saline (T0 to T13). Apocynin was given from T8 to T13. Group 6 (Dex+apocynin treatment): Rats were treated for 14 days with Dex (T0 to T13). Apocynin was given from T8 to T13.

Experiment 2: \(L\)-Arginine Reversal

In the \(L\)-arginine reversal study, groups were as follows. Group 7 (Saline treatment): Rats were treated with saline and fed sterile crushed feed for 14 days (T0 to T13). Group 8 (Dex treatment): Rats were treated with Dex (20 \(\mu\)g/kg/day subcutaneously) and sterile crushed feed for 14 days (T0 to T13). Group 9 (Saline+\(L\)-arginine treatment): Rats were treated for 14 days (T0 to T13) with saline and sterile crushed feed. \(L\)-arginine was mixed into food from T8 to T13. Group 10 (Dex+\(L\)-arginine treatment): Rats were treated for 14 days (T0 to T13) with Dex (20 \(\mu\)g/kg/day subcutaneously) and sterile crushed feed. \(L\)-arginine was mixed into food from T8 to T13.

At the end of the experiments, rats were killed under anaesthesia pentobarbitone (60 mg/kg intraperitoneally). Blood samples were collected from the right ventricle, and the thymus were isolated.

Measurements of SBP and BW

The SBP was measured at 9 to 11 AM on alternate days using a tail-cuff system \(^11\) (Narco Biosystems, Houston, TX). The animals were weighed after SBP measurement.

Thymus Weight

After the rats were killed, the thymus was isolated. Thymus weight was measured as a marker of glucocorticoid (GC) activity. Data were expressed as milligrams per 100 g body weight (BW).

Hematocrit

Hematocrit was measured only in the \(L\)-arginine reversal study. Blood was collected into capillary tubes to three
quarters full, sealed with putty, and centrifuged. Hematocrit was measured using the Hawksley reader (Hawksley & Son Ltd, Sussex, England). Data were expressed as percentage of cells per total volume of blood.

**Statistical Analysis**

Statistical analysis was performed using SPSS software, version 11.0 (SPSS Inc., Chicago, IL), using raw data. The Greenhouse-Geisser adjusted \( P \) values and \( P' \) values (the Bonferroni-corrected value) \( \leq .05 \) were regarded as significant. Data for SBP and BW were analyzed by repeated-measures analysis of variance and thymus weight, and hematocrit data were analyzed by unpaired \( t \) test. Data are expressed as mean \( \pm \) SEM.

**Results**

**Systolic BP**

**Apocynin Studies**

Saline treatment did not change SBP (113 \( \pm \) 4 mm Hg T12) while Dex significantly increased SBP from 104 \( \pm \) 3 mm Hg on T0 to 122 \( \pm \) 3 mm Hg on T12 (\( P < .01 \)). \( \Delta \)SBP between T12 and T0 was +5 \( \pm \) 5 mm Hg in saline-treated rats and +18 \( \pm \) 4 mm Hg in Dex-treated rats. SBP in Dex-treated groups was higher than saline controls (\( P' < .05 \)).

**Apocynin Prevention**

The SBP did not change significantly in apocynin+saline (115 \( \pm \) 3 mm Hg on T10) or apocynin+Dex treated rats (113 \( \pm \) 2 mm Hg T10) (Fig. 1A). Values of \( \Delta \)SBP between T0 and T10 were \(-10 \pm 5\) mm Hg in apocynin+saline and \(-6 \pm 2\) mm Hg in apocynin+Dex treated rats (Fig. 1B). In Dex-treated rats SBP was higher than in apocynin+Dex treated rats (\( P' < .01 \)). There was no significant difference in SBP between saline and apocynin+saline groups.

**Apocynin Reversal**

Before apocynin administration, SBP did not change (113 \( \pm \) 3 mm Hg T0 and 122 \( \pm \) 3 mm Hg T6, \( P = \) NS) in saline+apocynin treated rats but increased (from 116 \( \pm \) 3 mm Hg on T0 to 130 \( \pm \) 4 mm Hg on T6) in Dex+apocynin treated rats (\( P < .01 \)). Co-treatment with apocynin on day 8 did not change SBP in saline treated rats (120 \( \pm \) 3 mm Hg to 114 \( \pm \) 3 mm Hg on T12, \( P = \) NS) but decreased SBP in Dex-treated rats (from 132 \( \pm \) 4 mm Hg to 116 \( \pm \) 4 mm Hg on T12, \( P < .01 \)) (Fig. 2A). Values of \( \Delta \)SBP between T8 and T12 were \(-6 \pm 2\) mm Hg in saline+apocynin treated rats, and +6 \( \pm \) 5 mm Hg in Dex+apocynin treated rats (Fig. 2B). Before apocynin administration (T0 to T6), SBP was not significantly different between Dex and Dex+apocynin treated rats. After apocynin administration (T8 to T12), SBP in Dex-treated rats was higher than in Dex+apocynin (\( P' < .05 \)). SBP between saline and saline+apocynin treated groups was similar.

**L-Arginine Reversal**

In saline-treated rats, SBP was unchanged (122 \( \pm \) 3 mm Hg T0 and 118 \( \pm \) 1 mm Hg T12, \( P = \) NS). Dex significantly increased SBP from 122 \( \pm \) 3 mm Hg T0 to 132 \( \pm \) 4 mm Hg on T12 (\( P < .01 \)) (Fig. 3A). Value of \( \Delta \)SBP between T12 and T0 was \(-13 \pm 2\) mm Hg in saline-treated rats and +16 \( \pm \) 3 mm Hg in Dex-treated rats (Fig. 3B). Before L-arginine administration, SBP did not change (121 \( \pm \) 2 mm Hg and
119 ± 4 mm Hg (T6) in saline+L-arginine treated rats but increased in Dex+L-arginine treated rats (from 120 ± 3 T0 to 128 ± 2 mm Hg T6, P < .01). Co-treatment with L-arginine on day 8 had no effect on SBP in saline (124 ± 3 T8 and 118 ± 3 mm Hg T12, P = NS) or Dex-treated rats (126 ± 3 T8 and 133 ± 3 mm Hg T12, P = NS) (Fig. 3A). Value of ΔSBP between T8 and T12 were −4 ± 4 mm Hg in Dex-treated rats, and +7 ± 4 mm Hg in Dex+L-arginine treated rats (P = NS) (Fig. 3B). Compared with saline-treated rats, Dex-treated rats had higher SBP (P' < .01). L-arginine had no effect on SBP in either saline or Dex-treated rats.

**Change in BW**

The BW was increased in both saline-treated rats (from 252 ± 7 T0 to 318 ± 8 g T12, P < .01, Apocynin study; and from 262 ± 5 T0 to 301 ± 8g T12, P < .01, L-arginine study) and Dex-treated rats (from 238 ± 8 T0 to 267 ± 8g T12, P < .01, Apocynin study; and from 253 ± 3 T0 to 268 ± 4g T12, P < .01, L-arginine study). BW of saline-treated was higher than that of Dex-treated rats (P' < .01). Neither apocynin nor L-arginine changed BW in saline or Dex-treated rats.

**Water and Food Consumption**

Water consumption was 43 ± 1 mL/rat/day in saline and 36 ± 1 mL/rat/day in Dex-treated rats (P = NS). Apocynin did not affect daily water consumption. Food intake was 27 ± 1 g/rat/day in saline-treated rats and similar in Dex-treated and L-arginine–treated rats.

**Thymus Weight**

Treatment with Dex significantly decreased thymus weight (76 ± 4, Apocynin study; and 63 ± 5 mg/100 g BW, L-arginine study) compared with saline (147 ± 5, Apocynin study; and 137 ± 7 mg/100 g BW, L-arginine study) (P < .05). Neither apocynin nor L-arginine affected thymus weight (Fig. 4).

**Change in Hematocrit**

In the L-arginine reversal study, Dex increased hematocrit (57 ± 1%) compared with saline (52 ± 1%) (P < .01). L-arginine had no effect on hematocrit in Dex-treated (60% ± 1%) or saline-treated rats (51% ± 1%).

**Discussion**

Subcutaneous administration of Dex to male SD rats at doses from 1 to 10 μg/rat/day (~4 to 50 μg/kg/day Dex) increased SBP by 10 to 45 mm Hg in a dose-dependent manner. Administration of Dex at 10 μg/kg/day, ~2.5 μg/rat/day, to male SD rats for 13 days increased SBP (from 122 ± 5 to 136 ± 3 mm Hg) compared with saline. Decreases in thymus weight were observed in the Dex-treated group and weight gain was lower in Dex than in saline-treated rats consistent with known GC effects. In...
preliminary experiments for the present study, although Dex 10 μg/kg/day had demonstrable GC effects, SBP did not significantly increase (data not shown). However, Dex at 20 μg/kg/day (~5 μg/rat/day) significantly increased SBP, decreased thymus weight and decreased BW gain, compared with saline-treated rats, consistent with previous findings. Accordingly the apocynin and L-arginine experiments were performed at Dex 20 μg/kg/day. This dose of Dex is low, in contrast to the massive doses (0.25 to 10 mg/rat/day) often used by other workers, and minimizes deleterious metabolic effects; specifically, water intake was not increased at this dose, and animals continued to grow. This contrasts with the profound loss of weight that has been observed with higher doses of Dex.

Administration of Dex enhanced hydrogen peroxide production in human umbilical vein endothelial cells, and Dex (0.5 mg/kg/day) also increased xanthine oxidase (XO) levels in mesenteric arterioles in Wistar rats. Plasma F2-isoprostane (a marker of lipid oxidative stress) was significantly increased in SD rats treated with Dex, 10 μg/kg/day (which increased SBP in that study); and the SBP-raising effect of Dex was prevented and partially reversed by Tempol, suggesting that Dex-induced rise in SBP is associated with increased oxidative stress.

In the present study, apocynin prevented and reversed Dex-induced increase in SBP but had no effect on Dex-induced changes in body and thymus weight, suggesting that the antihypertensive effect of apocynin was independent of GC activity. Apocynin down regulates two important components of NAD(P)H oxidase, p22phox in rat aorta and p47phox in human endothelial cells. Concurrent administration with apocynin (1.5 mmol/L) and DOCA-salt to male SD rats for 21 to 28 days decreased BP and aortic superoxide production. The same dose of apocynin also completely prevented aldosterone–induced hypertension in male SD rats. Treatment with apocynin alone did not affect SBP. In the present study, treatment with apocynin at 1.5 mmol/L in drinking water reversed and prevented Dex (20 μg/kg/day)–induced increase in SBP in SD rats, while confirming that apocynin alone did not alter SBP. These results, together with our recent observations that apocynin also prevents and reverses ACTH-induced hypertension, indicate that NAD(P)H oxidase is a major enzymatic source of superoxide overproduction in rat model of both naturally occurring and synthetic GC-hypertension.

Xanthine oxidase is another important enzyme for ROS production. Incubation of human umbilical vein endothelial cells with Dex increased ROS production significantly and this effect was reversed by superoxide dismutase, an endogenous superoxide scavenger, showing that XO participated in the Dex-induced increase in superoxide production. In male Wistar rats, Dex (0.5 mg/kg/day, intramuscularly) increased XO levels in muscle, and allo-purinol, an XO inhibitor, prevented Dex-hypertension, suggesting that XO is also a source of ROS production in Dex-hypertension, at least at the very high doses used (500 μg/kg/day).

Uncoupled NOS (consequent to inadequate concentrations of the cofactor, tetrahydrobiopterin [BH4]) could also mediate superoxide rather than NO production. Significant inhibition in cytokine-stimulated generation of cellular BH4 and L-arginine uptake was observed in Dex-treated SD rat cardiac microvascular endothelial cells, and this decreased BH4 uptake may induce eNOS uncoupling. L-arginine enhanced superoxide generation in a BH4-free recombinant eNOS preparation. L-arginine has been reported to partially reverse Dex-suppressed acetylcholine-vasodilatation. However, we found that L-arginine did not prevent Dex-hypertension, although the same dose of L-arginine prevented and partially reversed adrenocorticotropic hormone (ACTH)–hypertension. This has been confirmed by Severino et al, who reported L-arginine did not prevent Dex (2 μg/rat/day subcutaneously)–induced hypertension in Wistar rats. In the present study, L-arginine did not modify Dex-hypertension. The fact that administration of L-arginine did not worsen Dex-hypertension in rats suggests that L-arginine does not increase superoxide production in Dex-hypertensive rats, and thus Dex does not induce uncoupled eNOS in this model. Further investigation of the effect of BH4 supplementation on Dex-hypertension is required to clarify this issue (eNOS uncoupling). In ACTH hypertension, such supplementation has no effect on SBP.

In contrast with the rat ACTH-hypertension and corticosterone-hypertension models, oral L-arginine did not reverse cortisol-hypertension in human beings. Cellular L-arginine transport was unchanged in patients with cortisol-hypertension. Exposing cardiac microvascular endothelial cells from SD rats to Dex significantly reduced L-arginine uptake and the cationic amino acid transporter (CAT)–1 (a selective L-arginine transporter) mRNA in rat cardiac microvascular endothelial cells and murine heart and cultured endothelial cells compared with controls. The fact that L-arginine had no effect on Dex-hypertension may reflect inhibition of L-arginine transport by Dex; however, we showed previously that plasma NOx concentrations were higher in rats concurrently treated with Dex and L-arginine compared with controls, implying that arginine can increase NOx in this model. In ACTH-hypertensive rats, plasma L-arginine concentrations were reduced compared with those in sham-treated rats. The effect of Dex on plasma L-arginine levels is yet to be investigated.

Hematocrit is a reliable indirect measurement of change in plasma volume. Administration of Dex 2 μg/rat/day (~8 to 10 μg/kg/day) to male SD rats raised hematocrit compared with control values. In the present study, Dex also significantly increased hematocrit consistent with previous findings, implying a decrease in rat plasma volume. This is compatible with the known glucocorticoid effects of increased water turnover.
In conclusion, Dex increased SBP and hematocrit, and decreased thymus and BW, compared with control values in SD rats. Apocynin prevented and reversed Dex-induced changes in SBP, suggesting that upregulation of superoxide production in Dex-hypertension is related to increased NAD(P)H oxidase activity. L-arginine did not modify established Dex-hypertension, suggesting that neither L-arginine deficiency nor eNOS uncoupling plays a critical role.

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