Inflammation compromises renal dopamine D1 receptor function in rats

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Asghar M, Chugh G, Lokhandwala MF. Inflammation compromises renal dopamine D1 receptor function in rats. Am J Physiol Renal Physiol 297: F1543–F1549, 2009. First published September 30, 2009; doi:10.1152/ajprenal.00366.2009.—We tested the effects of inflammation on renal dopamine D1 receptor signaling cascade, a key pathway that maintains sodium homeostasis and blood pressure during increased salt intake. Inflammation was produced by administering lipopolysaccharide (LPS; 4 mg/kg ip) to rats provided without (normal salt) and with 1% NaCl in drinking water for 2 wk (high salt). Control rats had saline injection and received tap water. We found that LPS increased the levels of inflammatory cytokines, interleukin-6, and tumor necrosis factor-α in the rats given either normal- or high-salt intake. Also, these rats had higher levels of oxidative stress markers, malondialdehyde and nitrotyrosine, and lower levels of antioxidant enzymes in gene expression events that impact cell

INFLAMMATION IS LINKED TO A WIDE VARIETY OF PATHOPHYSIOLOGICAL PROCESSES. SEVERAL STUDIES RECOGNIZE IT AS A CENTRAL MECHANISM CONTRIBUTING TO PROGRESSION OF CARDIOVASCULAR DISEASE (9, 32), HYPERTENSION (37, 39), DIABETES (15), AND MYOCARDIAL ISCHEMIA AND INFARCTION (32). THESE INFLAMMATORY STATES ARE CAUSED BY ORGAN TISSUE MALFUNCTION AND HOMEOSTASIS IMBALANCE OF ONE OR SEVERAL PHYSIOLOGICAL SYSTEMS. IT IS ASSUMED THAT THE CLASSIC INSTIGATORS OF INFLAMMATION “INFECTION AND INJURY” DO NOT PLAY A ROLE IN THE ETIOLOGIES OF THESE SITUATIONS (33).

THE TRANSCRIPTION FACTOR NF-κB IS A UBQUITOUS PROTEIN WHICH PLAYS A CRITICAL ROLE IN THE TRANSCRIPTION OF CYTOKINE GENES, SUCH AS TNF-α, IL-6, AND IL-1β, AND IN THE INFLAMMATORY PROCESSES (22, 27). MUCH OF OUR UNDERSTANDING OF NF-κB IS DERIVED FROM STUDYING IMMUNOLOGICALLY RELEVANT SIGNALING PATHWAYS. STUDIES IMPLICATE NF-κB IN GENE EXPRESSION EVENTS THAT IMPACT CELL SURVIVAL, DIFFERENTIATION, AND PROLIFERATION (22), WHEREAS ITS DYSREGULATION IS LINKED TO VARIOUS PATHOLOGICAL SITUATIONS.

Nuclear erythroid-related factor 1 and 2 (Nrf1 and Nrf2) belong to BZIP transcription factor family and are ubiquitously expressed. Nrf1 and in particular Nrf2 by trans activating anti-oxidant response element present in the promoter of genes encode enzymes involved in phase II detoxification and anti-oxidant defense (31). These anti-oxidant enzymes including superoxide dismutase (SOD) protect cellular damage and help maintain cellular homeostasis during oxidative stress.

Renal dopamine is an important regulator of blood pressure, sodium balance, and kidney function (3, 28). Dopamine exerts its effects via activation of D1-like and D2-like receptors, which belong to G protein-coupled receptor family (3, 28, 35). Out of these two receptor subtypes, D1-like receptor signaling cascade is linked to the inhibition of sodium transporters, Na-K-ATPase and Na/H exchanger in renal proximal tubules (RPTs) (3, 23, 25, 28, 35). It is the inhibition of these transporters in RPTs which promotes sodium excretion and contributes to the maintenance of sodium balance and blood pressure particularly during increases in sodium intake (23, 38).

Abnormalities in response to dopamine and in D1 receptor function have been implicated in the increase in blood pressure in hypertensive patients (40), in the elderly (52), and in rodent models of genetic and salt-sensitive hypertension (10, 11, 19, 24, 30, 38, 41). Both D1 receptor dysfunction and the development of hypertension have been linked to the increase in oxidative stress in these situations (2, 8, 47). However, these situations are also associated with inflammation (17, 34, 37, 39, 42). It is not known whether inflammation per se alters renal D1 receptor function. Therefore, the present study was designed to investigate the role of inflammation on D1 receptor function in RPTs of young Sprague-Dawley (SD) rats. Inflammation in SD rats was produced by administering lipopolysaccharide (LPS) and was confirmed by measuring inflammatory markers such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). NF-κB and Nrf2 involved in transcription of genes of inflammation and anti-oxidant enzyme, respectively, were also measured in the RPTs. Given the importance of renal D1 receptors in maintaining sodium homeostasis and blood pressure during increased salt intake, we measured blood pressure and markers of D1 receptor function in response to LPS in rats on normal- and high-salt intake. Furthermore, markers of oxidative stress, malondialdehyde (MDA) and protein nitrotyrosine, were also measured in the RPTs.

METHODS AND MATERIALS

Animals. Male SD rats (225–250 g) were purchased from Harlan (Indianapolis, IN) and allowed to acclimate for at least 5 days before any studies were conducted. They were fed commercial rat chow and water ad libium (unless specified) and housed in a temperature-, humidity-, and light-controlled (12:12-h light-dark cycle) environment in the University of Houston Animal Care Facility. The animals were used in the study with the approval of the Institution’s Animal Care and Use Committee and according to the National Institutes of Health guidelines.

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Animal treatment. The rats were randomly grouped as control, LPS, and high salt-LPS (HS-LPS) groups. The control and LPS rat groups were given tap water to drink, whereas HS-LPS rat group had 1% sodium chloride in drinking water for 2 wk. One day before death, the LPS and HS-LPS rat groups were administered with LPS (4 mg LPS in saline/kg body wt ip). The control rat group received only saline injection.

Blood pressure measurement. As described previously (8), rats were anesthetized with Inactin (100 mg/kg ip). Tracheotomy was performed to facilitate breathing. To measure blood pressure and to collect blood samples, the left carotid artery was catheterized with PE-50 tubing, and kidneys were perfused with collagenase and hyaluronidase. The kidneys were removed and kept in ice-cold oxygenated Krebs buffer containing (in mM) 1.5 CaCl$_2$, 110 NaCl, 5.4 KCl, 1 KH$_2$PO$_4$, 1 MgSO$_4$, 25 NaHCO$_3$, 25 d-glucose, and 2 HEPES (pH 7.6). Coronals sections of the kidneys were obtained, and superficial cortical tissue slices (rich in proximal tubules) were dissected out with a razor blade. The cortical slices were kept in fresh Krebs buffer. Enrichment of proximal tubules was carried out using 20% Ficoll in Krebs buffer. The band at Ficoll interface was collected and washed in Krebs buffer by centrifugation at 250 g for 5 min. Tubular cells’ viability was performed using the Trypan blue exclusion test.

Primary cultures from RPTs. The tubules isolated from the kidneys of control SD rats, as mentioned above, were washed with DMEM/F12 (1:1) culture media by centrifugation. The tubular pellet was used to culture epithelial cells as we described earlier (5) to study direct effect of LPS on Na-K-ATPase activity.

Preparation of proximal tubular membranes. RPTs were homogenized in sucrose buffer (in mM: 250 sucrose, 10 Tris, 1 PMSF, pH 7.5), and membranes were prepared using differential centrifugation method (4, 5).

D1 receptor numbers and D1 receptor proteins. To determine the number of D1 receptors on the membranes, binding of a D1 receptor antagonist 3H-SCH23390 to membranes was performed as described previously (4, 5). Briefly, for saturation binding, 50 µg of membrane proteins were incubated with 20 nM 3H-SCH23390 in a final volume of 250 µl binding buffer at 25°C for 90 min. Unlabeled SCH23390 (10 µM) was used for determining nonspecific bindings. Specific binding was calculated as the difference between the total and nonspecific bindings. D1 receptor protein was determined by Western blotting as described earlier (4, 5).

$[^{35}]S$-GTPγS binding assay. As previously described (4, 5), membrane proteins (5 µg) in the presence of $[^{35}]S$-GTPγS (0.6 nM corresponding to ~100,000 cpm) and GDP (10 µM) were incubated with various concentrations of SKF38393 (10$^{-10}$–10$^{-7}$ mol/l) in a final volume of 100 µl for 1 h at 30°C. Nonspecific binding was determined by adding 100 µM unlabeled GTP to the assay media. Specific binding was calculated as the difference between total and nonspecific bindings.

ELISA. TNF-α and IL-6 in the plasma and RPT homogenates were measured by ELISA according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN).

Na-K-ATPase assay. Na-K-ATPase activity in the RPTs was measured by determining phosphate released from ATP in the absence and presence of Na-K-ATPase inhibitor ouabain using a colorimetric method (33). $[^{86}]$Rubidium ($[^{86}]$Rb$^+$) uptake, an index of Na-K-ATPase activity, in primary cultures of RPTs was carried out according to our published method (5). Briefly, cells were incubated without ouabain (Na-K-ATPase activity) or with ouabain (Na-K-ATPase activity). The cells were lysed with 3% sodium dodecyl sulfate, and radioactivity was measured directly in cell lysate using a gamma counter. Na-K-ATPase activity was determined as the difference between $[^{86}]$Rb$^+$ uptake in the absence and presence of ouabain (1 mM).

Nuclear protein extraction. Nuclear proteins from RPTs were extracted using an extraction kit (Pierce, Rockford, IL) as described earlier (31). Transcription factors NF-κB and Nrf2 were determined in the nuclear proteins by Western blotting using specific primary rabbit NF-κB-p65 (34) and Nrf2 (4) antibodies. Nuclear histone-4 protein was probed using histone-4 antibody (Millipore, Temecula, CA) as a control for protein loading.

Measurement of MDA and nitrotyrosine. The levels of MDA and nitrotyrosine in the RPT homogenates were determined by colorimetric (4) and Western blotting (7) methods, respectively. Both MDA and nitrotyrosine are markers of oxidative stress. The levels of nitrotyrosine are measured by Western blotting, which help detect oxidative damage in different proteins in the same sample.

Measurement of SOD. SOD in the RPT homogenates was measured by Western blotting following our published method (4).

Protein measurement. Proteins were measured using BCA protein assay kit (Pierce) and BSA as standards.

Statistics. Results are presented as means ± SE. Data were analyzed by ANOVA or t-test followed by Newman-Keuls multiple comparison test wherever applicable. A value of $P < 0.05$ was considered significant.

RESULTS

As shown in Fig. 1, the levels of markers of inflammation TNF-α and IL-6 increased in LPS-treated compared with control rats. These markers also increased in response to LPS in rats with high-salt intake.

The levels of markers of oxidative stress, MDA (Fig. 2A), and protein nitrotyrosine (Fig. 2B) in the RPTs increased in LPS-treated compared with control rats. LPS also increased the
levels of these markers in the RPTs of rats given high salt. The levels of antioxidant enzyme SOD decreased in the RPTs in response to LPS in rats given normal and high salt (Fig. 2C).

The nuclear levels of transcription factor NF-κB involved in inflammatory processes increased (Fig. 3A), whereas transcription factor Nrf2 responsible for antioxidant defenses decreased (Fig. 3B) in the RPTs of LPS-treated rats given either normal or high salt.

The D1 receptor numbers (Fig. 4A) and D1 receptor proteins (Fig. 4B) decreased in the RPT membranes of LPS-treated rats irrespective of salt intake compared with control rats. The levels of D1 receptor proteins were not changed in the RPT homogenates among the three groups (Fig. 4C). The D1 receptor agonist SKF38393 (10⁻⁶ M) increased the binding of [³⁵S]GTPγS in the RPT membranes of control rats; however, it failed to increase the [³⁵S]GTPγS binding in the membranes of LPS-treated rats given normal or high salt (Fig. 5A). The basal activity of Na-K-ATPase in the RPTs was similar in control and LPS-treated rats irrespective of salt intake (control vs. LPS vs. HS-LPS: 105 ± 10 vs. 108 ± 13 vs. 97 ± 17 nmol·mg protein⁻¹·min⁻¹). Moreover, SKF38393 (10⁻⁶ M) decreased ⁸⁶Rb uptake in vehicle-treated epithelial cells, which was attenuated in cells pretreated with LPS (1 μg/ml, overnight; Fig. 5B).

As shown in Fig. 6, the systolic but not the diastolic blood pressure increased in response to LPS in rats with high-salt intake.

**DISCUSSION**

The present study clearly demonstrates that LPS produced inflammation (IL-6 and TNF-α) in SD rats and decreased the levels of Nrf2, a transcription factor involved in anti-oxidant...
enzyme gene expression, in the nucleus of RPTs. The abundance of an anti-oxidant enzyme SOD decreased while oxidative stress markers, MDA and nitrotyrosine, increased in the RPTs in response to LPS. The D1 receptor function reduced in the RPTs and in renal epithelial cells when LPS was administered in the animal or provided exogenously in the culture media, respectively. In response to LPS, systolic but not diastolic blood pressure increased in the rats with only high-salt intake. These results suggest that inflammation causes dysfunction of renal D1 receptors and is associated with high blood pressure in rats during salt overload.

Inflammation is generally characterized as an increase in the levels of systemic and organ tissue chemokines, cytokines (e.g., IL-6, TNF-α), and adhesive molecules (e.g., ICAM) (48). It increases in obesity, insulin resistance, essential hypertension, and aging (13, 37, 49). The dysfunction of renal D1 receptors in these situations contributes to increase in blood pressure (30, 38, 40, 51, 52). Therefore, there seems to be a strong association between inflammation and renal D1 receptor dysfunction. To study the effect of inflammation on D1 receptor function, we administered LPS, a bacterial membrane component, to SD rats. This is a commonly used model of inflammation, which has been helpful in understanding the mechanism of progression of renal diseases (40, 41). Higher doses (10 mg/kg) of LPS are reported to produce hypotension (46). In the present study, however, 4 mg/kg LPS administered by intraperitoneal injection did not alter blood pressure in the rats. Nevertheless, the LPS dose used in this study when given intravenously is reported to cause hypotension (14). The dis-
crepancy in the blood pressure between our and the above study may be due to the route LPS is administered (ip vs. iv).

LPS produces inflammation via NF-κB-mediated transcriptional regulation of chemokine and cytokine genes (27). We also found increased nuclear levels of NF-κB, an index of its activation, in the RPTs in response to LPS to a greater extent in rats given high salt than in rats given normal salt. While the reason for higher NF-κB activation in rats given high salt is not known, LPS increased the levels of inflammatory cytokines IL-6 and TNF-α in both groups of rats given normal and high salt. It should be mentioned that TNF-α detected in the RPTs in the present study is not from immune cells but from renal epithelial cells. This notion is based on the fact that 1) while isolating RPTs, immune cells are removed by perfusing the kidney and 2) renal epithelial cells are reported to produce cytokines in cultures (21).

There are studies suggesting interrelationship between inflammation and oxidative stress (45). LPS has been shown to increase reactive oxygen species (ROS) and inflammatory marker IL-1β and to decrease Cu/Zn SOD, and to cause renal dysfunction (50). We have also seen an increase in the levels of markers of oxidative stress in RPTs in response to LPS. In addition, in the present study, reduction in antioxidant enzyme Cu/Zn SOD levels and inhibition of the enzyme’s transcription factor Nrf2 were also found in RPTs. Nrf2 transcribes a battery of anti-oxidant enzyme genes including Cu/Zn SOD (12). While the study by Yang et al. (50) fails to provide a reason for the reduced Cu/Zn SOD levels, our study suggests that inactivation of Nrf2 during inflammation may have resulted in reduced Cu/Zn SOD levels in RPTs. This may have caused an imbalance in anti-oxidant capacity of RPTs leading to an increase in oxidative stress. Perhaps this is true since an exaggerated production of ROS in response to LPS was reported in peritoneal leukocytes isolated from Nrf2-deficient mice (43). Therefore, it seems that inflammation-induced downregulation of Nrf2 causes disturbances in the cellular anti-oxidant capacity, which may be a universal mechanism for inflammation-induced oxidative stress.

There are compelling pieces of evidence linking renal D1 receptor dysfunction and increase in blood pressure in obesity, insulin resistance, and hypertension (30, 38, 44). The direct evidence for D1 receptor in blood pressure regulation comes from the study where mice lacking D1 receptor gene had higher blood pressure (1). It is reported that the inability of dopamine to inhibit sodium transporter Na-K-ATPase is due to uncoupling of D1 receptors from G proteins, which may result in sodium retention and subsequently in an increase in blood pressure in spontaneously hypertensive and obese Zucker rats (26, 30). At the same time, these rat models are also associated with inflammation (39), which may be contributing to dysfunction of renal D1 receptors. The present study was an attempt to study the role of inflammation on D1 receptor function during normal and salt overload in SD rats. The purpose to include a salt overload rat group was to find changes in the blood pressure phenotype in them, if any, due to LPS-induced dysfunction of D1 receptors. In the present study, we did not include a rat group given only high salt. This was decided based on our previous findings that normal rats when given only high salt resemble vehicle-treated rats in terms of D1 receptor function and exhibit normal blood pressure (8).

It is interesting to note that LPS causes greater degree of oxidative stress, determined as MDA and nitrotyrosine, in RPTs of rats given high salt. This may be due to a cumulative effect of LPS and salt overload on oxidative stress as salt overload also is reported to increase oxidative stress (16). Probably, this is the reason for a profound decline in D1 receptor G protein coupling and in D1 receptor numbers in response to LPS in rats given high salt. However, a parallel
INFLAMMATION AND RENAL D1 RECEPTOR FUNCTION

change between D1 receptor numbers and D1 receptor proteins in the membranes in response to LPS in rats given high salt was not seen. This may be due to limitation in the sensitivity of the antibody-based (Western blotting) compared with the radioactive based (radioligand binding) methods. Radioligand binding assays are more sensitive than Western blotting.

Salt overload in normal SD rats is reported to decrease the basal activity of Na-K-ATPase in RPTs (8). However, in the present studies, high salt-induced reduction in Na-K-ATPase activity was not seen in LPS-treated rats. Moreover, D1 receptor agonist SKF38393 failed to inhibit Na-K-ATPase activity in renal epithelial cells in cultures prior treated with LPS. Taken together, these studies suggest an inability of D1 receptors to inhibit Na-K-ATPase and cause sodium excretion during inflammation and may have contributed to an increase in blood pressure in rats with high-salt intake. It should be noted that the effect of inflammation on blood pressure was studied after 24 h of LPS administration. We expect an even higher increase in blood pressure in high salt-fed rats with long-term LPS administration via osmotic pump, which needs to be determined.

We conclude that LPS induces inflammation, increases oxidative stress, and causes D1 receptor G protein uncoupling in RPTs. The LPS-induced dysfunction of renal D1 receptor may be associated with high blood pressure phenotype during salt overload. Furthermore, the increase in oxidative stress during inflammation may be due to downregulation of anti-oxidant capacity in RPTs.

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