Dopamine D3 Receptors and Salt-Dependent Hypertension

GERD LUIPPOLD,* CLAUDIA ZIMMERMANN,* MATTHIAS MAI,* DORIS KLOOR,* DOROTHEA STARCK,† GERHARD GROSS,† and BERND MÜHLBAUER*

*Department of Pharmacology, University of Tübingen, Tübingen; and †Department of Pharmacology, Knoll AG, Ludwigshafen, Germany.

Abstract. Alterations in the dopaminergic system may contribute to the pathogenesis of hypertension. Dopamine D3 receptors have been shown to be involved in the regulation of sodium balance and hemodynamics in rodents. For determining the role of D3 receptors in salt-dependent hypertension, clearance experiments were performed in anesthetized salt-sensitive (DS) and salt-resistant (DR) Dahl rats that were fed a standard diet with either normal (0.2%) or high (4%) sodium content for 21 to 26 d, which induced hypertension in DS but not in DR rats. The D3 receptor agonist R(+)-7-hydroxydipropyl-aminotetralin (7-OH-DPAT) increased GFR by up to 35% and urinary sodium excretion by up to 4.4-fold in DR rats that were on both normal and high-sodium diet. 7-OH-DPAT-induced natriuresis also was observed in DS rats that were on normal diet but not in hypertensive DS rats that were on high-salt diet. No GFR response to 7-OH-DPAT was found in DS rats, irrespective of sodium diet. The diminished functional response to D3 receptor stimulation in DS rats was associated with a significantly lower [3H]-7-OH-DPAT binding to renal membrane protein when comparing DS with DR rats. Consequently, DR rats were treated with BSF 135170, a novel, highly selective D3 receptor antagonist, for 29 d. Whereas no change in systolic BP was observed during normal diet, high sodium intake significantly increased BP by almost 40 mmHg. In summary, both expression and function of the renal dopamine D3 receptor are impaired in salt-sensitive Dahl rats. Together with the induction of salt-dependent hypertension in genetically salt-resistant Dahl rats by D3 receptor blockade, the data strongly suggest that the deficiency in dopamine D3 receptors represents an important pathophysiological factor in the development of salt-dependent hypertension.

Materials and Methods

All animal experimentation was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the German Law on the Protection of Animals. Male DS and DR Dahl rats that were aged 7 to 8 wk and weighed 125 to 175 g were purchased...
from M&B (Ry, Denmark). The animals were kept under controlled environmental conditions (21°C; 12-h dark/light cycle). According to the experimental protocol, the animals received ad libitum a standard rat diet (1324; Altromin, Lage, Germany) with either normal (0.2%) or a high (4%) sodium content for 21 to 26 d.

Clearance Experiments

The animals were anesthetized with thiopental sodium (80 mg/kg; Byk Gulden, Constance, Germany) and prepared for clearance study as described previously (15). In brief, catheters were inserted into the right jugular vein for infusion, into the carotid artery for blood sampling and monitoring of cardiovascular function, and into the bladder for urine collection. A tracheostomy was performed to keep the airways free. Via the first intravenous catheter, Ringer’s saline solution (in mmol/L: 111 NaCl, 30 NaHCO₃, 4.7 KCl) was infused at a rate of 0.8 ml/100 g body wt per h. Via the second catheter, [³H]-inulin (1.2 µCi/ml) dissolved in Ringer was infused at a rate of 0.6 ml/h throughout the entire experiment for assessment of GFR. The rats were allowed to recover from surgical procedures for 80 to 100 min before the measurements were started. Two consecutive clearance periods were carried out to obtain baseline values. Thereafter, Ringer infusion was switched to the specific D₃ receptor agonist R(+)-7-hydroxy-dipropyl-aminotetralin (7-OH-DPAT; Biotrend, Cologne, Germany), dissolved in Ringer, in two consecutive doses of 0.01 and 0.1 µg/(kg × min) while the infusion rate was maintained. Ten minutes after onset of each 7-OH-DPAT infusion, two clearance periods were performed. After the second period with 7-OH-DPAT administration, the infusion was switched back to the vehicle; after 10 min, another clearance period, designated as recovery, was carried out. Each clearance period comprised a 20-min urine collection, with a midpoint blood sample of 180 µl. An additional blood sample of 80 µl for the determination of plasma renin activity (PRA) was drawn during the second baseline period.

Radioligand Binding Studies

After normal or high-sodium diet of 21 d, rats were decapitated and the kidneys were removed rapidly through a midline abdominal incision and immediately placed on ice. Kidneys were freed of connecting tissue and homogenized in a motor-driven glass-Teflon Potter (Braun, Melsungen, Germany) with 1200 rpm for 4 strokes in buffer containing (mmol/L) Tris 25/HEPES 40 (pH 7.4), sucrose 320, ethylenediaminetetraacetate 0.5, and phenylmethylsulfonyl fluoride 0.01. The homogenate was centrifuged at 1000 x g for 15 min at 4°C (Biofuge; Heraeus, Hanau, Germany). The supernatant was discarded while the pellet was resuspended in the homogenization buffer and recentrifuged again (1000 x g, 15 min, 4°C). The second supernatant was centrifuged at 100,000 x g for 30 min at 4°C. Preliminary studies showed that this preparation was the most efficient for a high membrane/protein ratio (data not shown). The final pellet was suspended in the homogenization buffer in the absence of sucrose and ethylenediaminetetraacetate and stored at −80°C until binding experiments.

For binding studies, 100 µg of kidney membrane protein was used. The experiments were performed in buffer solution (pH 7.4) containing 25 mmol/L Tris and 40 mmol/L HEPES. Saturation assays were performed with 0.5 to 50 nmol/L [³H]-7-OH-DPAT (Amersham, Buckinghamshire, UK) and 10 µmol/L unlabeled 7-OH-DPAT for assessment of nonspecific binding. After incubation for 1 h at 20°C, the reaction was terminated by rapid filtration through glass-fiber filters (GF/B; Whatman, Maidstone, UK), presoaked in 0.3% polyethylenimine. Radioactivity adsorbed on the filters was determined by liquid scintillation counting. Specific binding was calculated as the difference between total binding and nonspecific binding. The conditions described derived from preliminary experiments: association studies were carried out at 0°C, 20°C, and 30°C. In these experiments, binding equilibrium was achieved at 20°C within 1 h. Thereafter, dissociation was started by adding 10 µmol/L unlabeled 7-OH-DPAT, showing a rapidly reversible 7-OH-DPAT binding. In addition, the dissociation constants (Kd values) estimated in these kinetic studies were similar to those calculated from the saturation experiments (data not shown).

Chronic Dopamine D₃ Receptor Inhibition

DR rats (n = 6) were treated for 29 d with BSF 135170 (Knoll AG, Ludwigshafen, Germany), a novel selective dopamine D₃ receptor antagonist (patent registration number WO 96/02520, priority date July 15, 1994). The high D₃ selectivity of this compound was demonstrated in competition studies using human dopamine D₂, D₃, and D₄ receptors expressed in human embryonic kidney cells, and native bovine caudate dopamine D₃ receptors as well. In comparison to a Kᵢ value of 13 mmol/L for the dopamine D₃ receptor, 50-fold, >800-fold, and >150-fold lower affinities were determined for the D₂, D₄, and D₃ receptors, respectively.

BSF 135170 was dissolved in 40 µl of acetic acid (100%) and added to 500 ml of drinking water. The concentration was adjusted daily to achieve a dose of 40 mg/kg per d. The animals were kept on normal rat diet for the first week, and tail-cuff BP measurements were performed on days 0 and 7 of BSF 135170 treatment. After the BP measurement on day 7, the high-sodium diet was initiated and BP was assessed on days 8, 15, 22, and 29 of the study. DS and DR rats that were on high-sodium diet without drug treatment (only vehicle in the drinking water) served as controls for salt sensitivity and salt resistance, respectively.

Analytical Procedures

Sodium concentrations were determined by flame photometry (ELEX 6361; Eppendorf, Hamburg, Germany). The [³H]-inulin and the [³H]-7-OH-DPAT radioactivity were measured by liquid phase scintillation counting (2550 TR; Canberra-Packard, Frankfurt, Germany). PRA was determined by its capacity to generate angiotensin I with addition of renin substrate, as described previously (17). The protein concentration was determined using Coomassie blue and bovine serum albumin as a standard according to the method of Bradford (18).

Statistical Analyses

Dissociation constant and maximum receptor density in the binding experiments were calculated using LIGAND (19) and nonlinear regression analysis. In clearance experiments, renal excretory and hemodynamic values were calculated per gram of kidney wet weight. Results were expressed as mean ± SEM. Differences within and between groups were compared using the ANOVA with Bonferroni correction. P < 0.05 was considered statistically significant.

Results

Clearance Experiments

As shown in Table 1, mean arterial BP (MAP) of DS and DR rats that were on normal sodium did not differ under anesthesia. When animals were fed the high-sodium diet, DS rats developed hypertension, whereas MAP did not change in DR animals. Heart rate was similar when comparing DS and DR rats. Infusion of the dopamine D₃ receptor agonist 7-OH-
DPAT slightly (by 5%) lowered BP and heart rate with no significant differences when comparing DS and DR rats as well as the diets. Body weight was significantly higher in DS than in DR rats, on both normal and high-sodium diet. Baseline sodium plasma concentration, however, did not significantly differ among all groups (Table 1). On high-sodium diet, kidney weight corrected for body weight was significantly greater in DS than in DR rats, whereas no difference was observed on normal sodium diet. As expected, PRA, which was measured at baseline, was significantly lower during high-sodium intake as compared with normal diet. However, there were no significant differences in PRA when comparing DS and DR rats (Table 1).

At baseline, GFR and urinary flow rate (UV) were slightly but not significantly lower in DS than in DR rats. The high-sodium condition significantly decreased GFR and UV in both DR and DS rats (Table 1). No significant difference in absolute or fractional sodium excretion (\(U_{NaV}\) and \(FE_{Na}\), respectively) occurred in DS as compared with DR rats (Table 1). Both values were moderately but not significantly higher in rats that were on the high-sodium diet. Infusion of 7-OH-DPAT in DR rats that were on normal sodium significantly increased GFR by up to 10% and UV by up to 1.8-fold. These effects were even more pronounced during the high-salt diet (Figure 1). Both GFR and UV tended to return to baseline after discontinuation of the 7-OH-DPAT infusion. In contrast to DR rats, there was no 7-OH-DPAT-induced increase in GFR in animals of the DS strain, irrespective of sodium diet (Figure 1). Similarly, the UV response to 7-OH-DPAT in DS animals was attenuated during the normal and absent during the high-sodium condition (Figure 1). In DR rats that were fed the normal sodium diet, 7-OH-DPAT dose-dependently increased \(U_{NaV}\) and \(FE_{Na}\) by up to 4.4-fold and 3.4-fold, respectively, whereas these effects were less pronounced during the high-sodium intake (Figure 2). 7-OH-DPAT elevated \(U_{NaV}\) and \(FE_{Na}\) also in DS rats that were on normal diet. However, this effect was absent on the high-sodium diet (Figure 2).

**[\(^3\)H]-7-OH-DPAT Binding**

Data of the binding experiments are summarized in Figure 3. Affinity of [\(^3\)H]-7-OH-DPAT to kidney membrane preparations as expressed by dissociation constant (\(K_d\)) values averaged approximately 10 nmol/L and did not differ when comparing DS and DR rats both on normal and high-sodium diet. In contrast, maximum density of binding sites (\(B_{max}\)) was significantly lower in DS when compared with DR animals. It is interesting that high-sodium diet decreased \(B_{max}\) of [\(^3\)H]-7-OH-DPAT binding to kidney membrane preparations by 26% and 50% in DR and DS rats, respectively. Consequently, the differences between DR and DS with respect to \(B_{max}\) were even more pronounced under high-sodium intake.

**BP Measurements**

The time course of systolic BP in conscious DS and DR rats that were on normal and high-sodium diets as well as on dopamine D3 receptor blockade by BSF 135170 is illustrated in Figure 4. During normal sodium diet (first week), the mean values of DR rats did not differ when comparing BSF 135170 and vehicle treatment. In DS rats, BP was significantly higher in comparison with DR animals. When the high-sodium diet was initiated, BP increased immediately by 10 to 15 mmHg in all groups. In DR rats that were on vehicle treatment, BP returned to baseline within 14 d of the sodium load. In contrast, BP in DR rats that were treated with BSF 135170 further increased during the high-sodium diet to a maximum increase of approximately 40 mmHg on day 29. In DS rats, systolic BP rose by the same extent. However, because of the higher baseline values, the sodium-induced hypertension on study day 29 was more pronounced in these animals.

**Discussion**

The significance of the dopaminergic system in the pathophysiology of arterial hypertension, although suggested decades ago, still has to be elucidated. In the present study,
pharmacologic blockade of dopamine D₃ receptors conferred salt sensitivity to DR rats. In addition, an attenuated renal response to D₃ receptor activation was observed in DS rats. Thus, this study provides strong functional evidence that deficient dopamine D₃ receptors are involved in the development of salt-dependent hypertension.

Transplantation of kidneys from DS to DR rats can lead to salt-induced hypertension, and, conversely, DR kidneys that are transplanted into DS rats can normalize BP, indicating that renal dysfunction contributes to hypertension in this model (20). Several lines of evidence indicate that hypertension in DS rats results from the inability to excrete sodium adequately in response to a salt challenge (21). It was proposed that the retention of sodium and the consecutive expansion of extracellular volume triggers mechanisms that affect BP, e.g., cardiac output, total peripheral resistance, activity of the sympathetic nervous system (22,23). However, the underlying mechanisms are unsatisfactorily understood. Several neuroendocrine systems, because of their role in the regulation of sodium excretion, might be involved in the cause of Dahl hypertension. In this context, the renin-angiotensin system was proposed because plasma and renal renin activity were lower in DS than in DR rats (24). In contrast, PRA in the present study did not differ between DS and DR rats but responded appropriately to

Figure 1. Effect of 7-OH-DPAT on GFR (A) and urinary flow rate (UV; B) in Dahl salt-resistant (DR) and Dahl salt-sensitive (DS) rats that were fed either normal (NS) or high-sodium (HS) diet. Depicted is the absolute change related to baseline values (Table 1) in GFR and UV in response to infusion of two different doses of 7-OH-DPAT or Ringer solution (REC). Values represent means ± SEM; n = 8 to 10 per group. †P < 0.05 versus baseline (see Table 1); *P < 0.05 versus respective DR rats.

Figure 2. Effect of 7-OH-DPAT on absolute urinary sodium excretion (U₅Na; A) and fractional excretion of sodium (FE₅Na; B) in anesthetized Dahl rats. See Figure 1 for further abbreviations.
changes in sodium intake. This observation does not support the idea that renin is involved in the pathogenesis of Dahl hypertension, however, with the limitation that renal renin was not assessed in the present study.

Abnormalities in the renal dopaminergic system, in view of its potential natriuretic actions, were suggested to contribute to the development of salt-dependent hypertension. Transgenic mice that lack one or both D1 alleles developed high BP (12). In addition, a defective coupling of the D1-like receptor was suggested in DS rats: D1-like receptor agonists were unable to stimulate adenylate cyclase in proximal tubules of kidneys from DS rats, whereas forskolin, as a direct cyclase activator, stimulated the enzyme in both DS and DR rats (25).

In vivo evidence for a defective dopamine D1 receptor in prehypertensive DS rats was reported by Hansell (26). More recently, also derived from observations in a transgenic mouse model, deficiency of the D3 receptor was suggested to cause hypertension (16). However, the pathophysiology induced by disruption of D1 or D3 receptors seems to be clearly different because the renal renin activity was much greater in mice that lack D3 receptors in comparison to wild-type controls (16), whereas dopamine D1 receptor activation has been reported to increase renin release (27).

In the present study, the renal response of DR and DS rats to 7-OH-DPAT was significantly different. Regarding the relatively high structural homology between the D2 and the D3 receptor protein, one might question the selectivity of 7-OH-DPAT as a D3 agonist. However, binding experiments showed a 100-fold D3-over-D2 selectivity of 7-OH-DPAT (28). In a recent study, 7-OH-DPAT-induced changes in renal function in normotensive Sprague-Dawley rats were attenuated by U-99194A, a selective D3 antagonist, but not by the D2 receptor blocker S(-)-sulpiride (15). The 7-OH-DPAT-induced changes in GFR observed in that study also were seen in the

Figure 3. Original saturation curves of [3H]-7-OH-DPAT binding to membrane protein of kidneys from DR and DS rats that were fed NS diet (A and C, respectively). Preparations were incubated with the radioligand alone (■), or plus unlabeled 7-OH-DPAT (▲) to define the unspecific binding. Specific binding (●) was calculated by subtracting unspecific from total binding. (B) The Bmax values of specific binding of [3H]-7-OH-DPAT in Dahl rats on NS or HS diet. Numbers indicate the respective dissociation constants (Kd). Values are means ± SEM; n = 10/4 per group. *P < 0.05 versus respective Dahl strain fed NS; †P < 0.05 versus respective DR rats (ANOVA).

Figure 4. Effect of the selective D3 receptor antagonist BSF 135170 on systolic BP of conscious DS and DR rats that were kept on NS (0.2%) diet from day 0 to day 7 and on HS (4%) diet from day 8 to day 29. Values are means ± SEM; n = 6 per group. *versus baseline value (day 0); †versus DR rats on tap water (ANOVA).
present experiments but only in DR rats, whereas in DS rats virtually no effect on GFR occurred. This seems to be the more relevant because the unstimulated GFR in DS rats was low rather than high compared with DR rats, excluding the possibility that an increased baseline level limited the capacity to increase GFR further. In good correspondence with the present observations, the typical rise in GFR in response to amino acid infusion was markedly impaired in isolated kidneys from prehypertensive DS rats when compared with kidneys from DR rats (29). Taken together, the data suggest that as a dominant consequence of dopamine D3 receptor deficiency, regulation of renal and/or systemic hemodynamics is altered in DS rats.

The experiments showed, in addition, that the tubular response to 7-OH-DPAT, i.e., the increase in urinary sodium excretion, clearly was impaired in DS when compared with DR rats. However, this holds true only for hypertensive rats of the DS strain that were on a high-sodium diet, whereas in prehypertensive DS rats that were on normal sodium intake, the tubular excretory response to D3 receptor activation was not affected. Even if these functional variations were paralleled by changes in [3H]-7-OH-DPAT binding, the obliging hypothesis that in the Dahl model of salt-dependent hypertension a deficient dopamine D3 receptor expression, via an impaired tubular electrolyte handling and consequently sodium retention, leads to extracellular volume expansion and thus to systemic arterial hypertension seems to be somewhat simplifying.

The D3 receptor density in kidneys of DS rats, as indicated by [3H]-7-OH-DPAT binding, was reduced in comparison with DR rats, whereas receptor affinity was not altered. In contrast to the present data, Barili et al. (30) reported 10- to 20-fold lower Kd values for binding of [3H]-7-OH-DPAT to sections of rat renal cortex. This might be due to differences in the tissue preparation or to experimental details, e.g., unspecific binding. Because the binding kinetics and the saturation experiments of the present study delivered almost identical Kd values and reasonable characteristics of the unspecific binding, the Bmax values seem to be plausible. Because, so far, no other data have been reported on D3 receptor affinity in renal tissue, additional studies in this context are desirable. In accordance with the present data on [3H]-7-OH-DPAT binding, we found recently that renal dopamine D3 receptor mRNA expression was diminished to the same extent in animals of the DS strain when compared with DR rats (31).

The marked reduction of dopamine D3 receptors induced by high-salt diet, as indicated by the binding experiments, was unexpected and difficult to explain. One might suggest that this obvious downregulation might depend on an increased presence of intrarenal dopamine as a result of elevated synthesis in response to the sodium load as was proposed by several authors (for review, see reference 32). However, an increase in tubular dopamine release in response to high sodium could not be confirmed in more recent studies in rats (4) and humans (33). In DS rats, total renal dopamine tissue levels were not increased as a result of a high-sodium diet (34), even though a lower renal dopamine content was reported in DS when compared with DR rats (35). Taking these observations together, an elevated renal dopamine production seems to be unlikely to explain the receptor downregulation observed. It should be noted that the high-sodium diet induced a reduction of renal D3 receptors only partly, whereas the tubular response to 7-OH-DPAT in DS rats was absent, which might indicate that functional inactive D3 receptors were detected by the binding experiments. One might speculate that internalization of D3 receptors from the cell membrane as shown previously for adrenergic receptors (36) was responsible for this phenomenon. It has not been determined, however, whether the receptor targeting and recycling to and from the cell membrane also are applicable to the D3 receptor.

The present data clearly support the idea of an involvement of renal dopamine D3 receptors in the tubular sodium handling. Unclear, however, are the different effects of 7-OH-DPAT on GFR when comparing DS rats that are on normal sodium diet and DR rats with high-sodium intake, which seem to have similar densities of renal D3 receptors. Even if not evaluated in this study, additional defects in the linkage of D3 receptors to second messengers or abnormal sodium transporters in DS rats as well might contribute to functional differences. In the proximal convoluted tubule of DS rats that were under low- and high-sodium intake, the D1 agonists fenoldopam and SND-919-C12 did not stimulate adenylate activity, indicating a defective signal transduction of the D1 receptor (25). In view of a potential interaction of D1- and D2-like receptors to inhibit sodium transport as was suggested previously (37), other receptors may act in concert to compensate for the loss of function if one of these subtypes is impaired. Such mechanisms might be overcome if the expression of the receptors involved is significantly reduced.

Nonetheless, striking evidence for the involvement of an impaired sodium handling in the pathogenesis of hypertension as a result of D3 receptor deficiency derives from the induction of salt-dependent hypertension in genetically salt-resistant Dahl rats by chronic treatment with a selective D3 antagonist. Even if these rats did not reach the same level of hypertension as did rats of the DS strain, their net BP increase was identical. In this context, Shigetomi et al. (38) reported that the unsselective D2-like antagonist metoclopramide induces hypertension in salt-loaded Wistar rats possibly via inhibition of endogenous Na+/K+-ATPase. In good accordance with our experiments, this study indicates that dopaminergic receptors of the D2-like family are involved in controlling BP and sodium handling of the kidney. The high selectivity of the pharmacologic tools used in the present study, however, clearly points toward the D3 type of this receptor family as the major pathologic principle.

To summarize, the data suggest that both expression and function of the renal dopamine D3 receptor are decreased in salt-sensitive Dahl rats. The idea that this deficiency contributes to the pathogenesis of hypertension is supported by the phenotypical conversion of salt-resistant into salt-sensitive Dahl rats by pharmacologic receptor inhibition. Because both renal vascular regulation and tubular excretory function are affected by the dopamine D3 receptor deficiency, additional investigational effort is needed on the exact pathomechanisms
and on the possibility that additional factors independent of the obvious receptor defect are involved.

Acknowledgments
This study was supported by the Deutsche Forschungsgemeinschaft (DFG, Mu 1297/1-2) and the Federal Ministry of Education, Science, Research and Technology (BMBF, 01EC9405).

We thank Dr. Margitta Albinus for the measurement of PRA and Dr. Swetlana Schneider for helpful support in the clearance experiments. The technical assistance of Christine Piesch also is appreciated.

References
15. Luippold G, Küster E, Joos TO, Mühlbauer B: Dopamine D3 receptor activation modulates renal function in anesthetized rats. Naunyn-Schmiedeberg’s Arch Pharmacol 358: 690–693, 1998
27. Yamaguchi I, Yao L, Sanada H, Ozono R, Mouradian MM, Jose PA, Carey RM, Felder RA: Dopamine D1A receptors and renin release in rat juxtaglomerular cells. Hypertension 29: 962–968, 1997