The 5-Hydroxytryptamine$_{2A}$ Receptor Is Involved in (+)-Norfenfluramine-Induced Arterial Contraction and Blood Pressure Increase in Deoxycorticosterone Acetate-Salt Hypertension

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ABSTRACT

The highly effective anorexigen (+)-fenfluramine was widely used to control body weight until the association with primary pulmonary hypertension and valvular heart disease. (+)-Norfenfluramine is the major hepatic metabolite of (+)-fenfluramine and is primarily responsible for the anorexigen effect as well as side effects. We reported that (+)-norfenfluramine causes vasoconstriction and a blood pressure increase in rats with normal blood pressure via the 5-hydroxytryptamine (5-HT)$_{2A}$ receptor. With the knowledge that (+)-norfenfluramine also has affinity for 5-HT$_{2B}$ receptors and that arterial 5-HT$_{2B}$ receptor expression is up-regulated in deoxycorticosterone acetate (DOCA)-salt hypertension, we tested the hypothesis that (+)-norfenfluramine-induced vasoconstriction and pressor effects are potentiated in DOCA-salt hypertensive rats in a 5-HT$_{2B}$ receptor-dependent manner. Contractions of arteries were measured using an isolated tissue bath system or myograph. Mean arterial blood pressure was measured in chronically instrumented conscious rats. Effects of (+)-norfenfluramine in stimulating arterial contraction (leftward shift versus SHAM, aorta, 5.13-fold; renal artery, 1.95-fold; mesenteric resistance artery, 1.77-fold) and raising blood pressure were significantly enhanced in hypertension. In arteries from both normotensive and hypertensive rats, (+)-norfenfluramine-induced contraction in aorta was inhibited by 5-HT$_{2A}$ receptor antagonists, ketanserin and LY53857 (4-isopropyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl)4,6,6-octahydroindolo[4,3-fg]quinoline), but not by the 5-HT$_{2B}$ receptor antagonist, LY272015 [6-chloro-5-methyl-N-(5-quinolinyl)-2,3-dihydro-1H-indole-1-carboxamide]. Ketanserin (3 mg/kg) reduced (+)-norfenfluramine-induced pressor response in both SHAM and DOCA rats. Our results demonstrate that (+)-norfenfluramine-induced arterial contraction and blood pressure increases are potentiated in DOCA-salt hypertensive rats. However, it is the 5-HT$_{2A}$ receptor and not the 5-HT$_{2B}$ receptor that participates in these effects.

The highly effective anorexigen (+)-fenfluramine (Redux) was taken off the market in 1997 because of the incidence of primary pulmonary hypertension (Abenhaim et al., 1996) and aortic valvular disease (Connolly et al., 1997). The anorexigen effect of (+)-fenfluramine is due to activation of 5-hydroxytryptamine (5-HT)$_{2C}$ receptor (Smith et al., 2006), whereas the 5-HT$_{2B}$ receptor plays an important role in the pathogenesis of (+)-fenfluramine-induced pulmonary hypertension (Launay et al., 2002) and aortic valvular disease (Fitzgerald et al., 2000). Radioligand binding experiments show that (+)-fenfluramine has low affinity for the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors, but the hepatic deethylated metabolite of (+)-fenfluramine, (+)-norfenfluramine, has high affinity at the 5-HT$_{2B}$ receptor ($K_i = 11.2$ nM), 5-HT$_{2C}$ receptor ($K_i = 324$ nM) and a lesser affinity at the 5-HT$_{2A}$ receptor ($K_i = 1516$ nM) (Rothman et al., 2000). Consistent with binding results, two studies showed that (+)-norfenfluramine causes more severe vasoconstriction by activation of 5-HT$_{2A}$ receptors in pulmonary arteries (Hong et al., 2004) and systemic arteries (Ni et al., 2004a) compared with effects of (+)-fenfluramine in arteries from normal rats. Studies in cells expressing recombinant human 5-HT$_{2B}$ receptors demonstrated that (+)-norfenfluramine is a 5-HT$_{2B}$ receptor agonist because

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; DOCA, deoxycorticosterone acetate; PSS, physiological salt solution; PE, phenylephrine; PBS, phosphate-buffered saline; RT, reverse transcriptase; PCR, polymerase chain reaction; LY53857, 4-isopropyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl)4,6,6-octahydroindolo[4,3-fg]quinoline; LY272015, 6-chloro-5-methyl-N-(5-quinolinyl)-2,3-dihydro-1H-indole-1-carboxamide; C$_T$, threshold cycle.
(+)-norfenfluramine potently stimulated the hydrolysis of inositol phosphates, increasing intracellular Ca\(^{2+}\), and activating the mitogen-activated protein kinase cascade (Fitzgerald et al., 2000). Our previous experiments showed that (+)-norfenfluramine induced arterial vasoconstriction by activating 5-HT\(_{2A}\) receptors but not 5-HT\(_{2B}\) receptors in normal rats. However, because the 5-HT\(_{2B}\) receptor is up-regulated and plays an important role in mediating 5-HT-induced arterial contraction and elevated blood pressure (Watts and Fink, 1999) in deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Watts et al., 1996; Watts, 2002), we hypothesized that (+)-norfenfluramine-induced vasoconstriction and pressor responses would be increased due to activation of an up-regulated 5-HT\(_{2B}\) receptor.

In this study, we first investigated whether (+)-norfenfluramine was a more potent contractile agonist in various isolated arteries from DOCA-salt rats when compared with SHAM rats. We then investigated the receptor mechanism of (+)-norfenfluramine-induced contraction in aorta from normotensive and DOCA-salt hypertensive rats by using 5-HT receptor antagonists. Lastly, we performed in vivo experiments to compare an acute (+)-norfenfluramine-induced response in normotensive and hypertensive rats.

Materials and Methods

Animal Use

Normal male Sprague-Dawley rats (0.225–0.300 kg; Charles River, Portage, MI) were used in the experiments.

Model of Hypertension

Rats were uninephrectomized and DOCA (200 mg/kg in silicone rubber) pellets were implanted s.c. Postoperatively, the rats were given a solution of 1% NaCl and 0.2% KCl for drinking. SHAM rats were uninephrectomized, received no DOCA, and drank normal tap water. All rats were given free access to standard pelleted rat chow (Harlan Teklad 8640 rodent diet; Harlan Teklad, Madison, WI). Animals remained on this regimen for 4 weeks before use.

Blood Pressure Measurement for in Vitro Studies

Systolic blood pressures of rats were determined in the conscious state by the tail cuff method (pneumatic transducer; Narco, Lewisville, TX).

Isolated Tissue Bath Protocol

Thoracic aorta, renal artery and stomach fundus were removed and placed in normal, physiological salt solution (PSS) containing 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH\(_2\)PO\(_4\), 1.17 mM KH\(_2\)PO\(_4\), 1.6 mM CaCl\(_2\), 2H\(_2\)O, 14.9 mM NaHCO\(_3\), 5.5 mM dextrose, and 0.03 mM Ca\(_{2+}\)EDTA, pH 7.2. Vessels were trimmed of fat, cut into helical strips (aorta, 2 × 10 mm; renal artery, 1 × 5 mm). Two longitudinal strips were made from each fundus. In experiments comparing responses of arteries from normotensive and hypertensive rats, an arterial strip from a DOCA-salt hypertensive and a SHAM normotensive rats were mounted in the same tissue bath.

Tissues were attached to a fixed, stainless steel rod at one end and to a force transducer at the other. Baths were filled with PSS, warmed to 37°C, and aerated with 95% oxygen and 5% carbon dioxide. Each tissue was placed under optimal resting tension (previously determined 1500 mg for the rat aorta, 500 mg for renal artery, and 4000 mg for stomach fundus) and allowed to equilibrate for 1 h with frequent buffer changes. Arterial tissues were then challenged with a maximal concentration of the \(\alpha_1\) adrenergic agonist phenylephrine (PE; 10 \(\mu\)M), or fundi were challenged with 67 mM KCl to initiate a maximal contraction and washed repeatedly until tone returned to baseline. To examine the status of the arterial endothelium, tissues were contracted with a half-maximal concentration of PE (10–100 nM), and once the contraction plateaued, the muscarinic agonist acetylcholine (1 \(\mu\)M) was administered. We considered a relaxation to acetylcholine greater than 60% of the PE-induced contraction as endothelium-intact tissue. Tissues were again washed until baseline was reached, and then one of the following protocols was followed.

Protocol 1: Testing of (+)-Norfenfluramine as Contractile Agonists in Tissues from SHAM and DOCA-Salt Rats. Concentration response curves to (+)-norfenfluramine (10 nM to 300 \(\mu\)M for rat aorta and superior mesenteric artery or 1 nM to 30 \(\mu\)M for renal artery) were performed in a cumulative manner. Each concentration was incubated for a minimum of 3 min; when contraction reached a maximum, the next higher concentration of agonist was added. Contraction to agonist was normalized to maximal contraction to PE in arterial tissues or KCl in fundi.

Protocol 2: Testing of Effect of Antagonists on (+)-Norfenfluramine-Induced Contraction. Vehicle or an antagonist was added to the bath. Antagonists were incubated with the tissues for 1 h, at which time the cumulative response to (+)-norfenfluramine in the presence of vehicle (control), or an antagonist was examined. All experiments testing the effect of an antagonist were paired with control (+)-norfenfluramine concentration curves in tissues from same rats. Tissues were not exposed twice to (+)-norfenfluramine.

Myograph Protocol. The whole intestine was placed in a silastic-filled Petri dish and pinned down. Second and third order arteries (200–300 \(\mu\)m, inner diameter) were carefully dissected and placed in cold PSS. Two tungsten wires (California Wire Products Corporation, Corona, CA) were threaded through the lumen of the artery. One wire was mounted to a micrometer and the other to a microtransducer. Arteries from SHAM and DOCA-salt animals were mounted at the same time. All arteries were examined with an intact endothelium. Baths, filled with PSS, were warmed by a water-circulated jacket around the bath and were aerated with 95% \(O_2\) and 5% \(CO_2\). Tissues equilibrated for 30 min before an optimal passive tension of 400 mg (determined previously) were applied using the micrometer. Tissues equilibrated another 30 min with frequent buffer changes prior to challenge with a maximal concentration of PE (10 \(\mu\)M). Experiment protocols were as described above.

Immunohistochemistry

Thoracic aorta tissues were cleaned of fat and fixed 24 h in 10% formalin. Tissues were paraffin embedded, and 5-\(\mu\)m sections were collected onto clean glass slides. Sections were dried overnight at 37°C. Slides were dewaxed in xylenes (two washes, 3 min each) and then were rehydrated by passing through graded alcohols (two changes 100% ethanol, 3 min each, followed by two changes 95% ethanol, 3 min each). Slides were rinsed in deionized water and air dried before use. Slides were immersed in Antigen Unmasking Agent (Vector Laboratories, Burlingame, CA) and microwaved on high for 5-min intervals two to three times and were then allowed to cool in the unmasking solution. Slides were rinsed twice in deionized water before proceeding. Endogenous peroxidases were blocked by incubating samples with 0.3% hydrogen peroxide in phosphate-buff ered saline (PBS) for 30 min. Sections were blocked for nonspecific binding in PBS containing 1.5% competing serum. In a humidified chamber, samples incubated overnight with a 5-HT\(_{2A}\) receptor antibody (Immunostar, Inc., Hudson, WI; 4°C, 5 \(\mu\)g/ml with 1.5% blocking serum in PBS), 5-HT\(_{2A}\) receptor antibody plus 5 \(\mu\)g/ml 5-HT\(_{2A}\) receptor antibody plus \(5 \times 10^{-5}\) M 5-HT\(_{2A}\) receptor antibody, or only blocking serum as a control. Then samples were washed three times with PBS and incubated with a peroxidase-conjugated secondary antibody (30 min, room temperature). Samples were washed and incubated with Vectastain Elite for 30 min and then incubated with 3,3-diaminobenzidine/H\(_2\)O\(_2\). Reactions were stopped with washing, counterstained with Vector hematoxylin, and slides were air dried, mounted, coverslipped, and slides were photo-
graphed using an inverted Nikon microscope with a digital camera (Nikon, Tokyo, Japan).

Real-Time RT-PCR
Two-step real-time RT-PCR was performed using a GeneAmp 7500 Real Time PCR machine (Applied Biosystems, Foster City, CA). Total RNA was isolated using standard TRIzol procedures (Invitrogen, Carlsbad, CA), reverse-transcribed, and then taken through standard real-time RT-PCR. Rat β-actin primer was purchased from SuperArray Bioscience Corporation (Frederick, MD). The primers for rat 5-HT2A receptor were designed as follows: 5-HT2A receptor forward, 5′-CTG ATG ATG TGC TGC TGG GTT TCC TTG TC-3′; and 5-HT2A receptor reverse, 5′-AGG TGC ATG ATG GAT GCC GCA GAA-3′. The product was a 141-bp amplicon. PCR conditions were: 95°C for 10 min for AmpliTaq activation, 15 s at 95°C, and 60 s at 60°C. Twenty microliters of the amplified products was run on a 3% agarose gel and stained with ethidium bromide. Bands on the gel were visualized using a Bio-Rad Fluor-S (Bio-Rad, Hercules, CA).

In Vivo Experiments
This surgery took place 3 weeks after DOCA or SHAM surgery. Catheters were constructed of polyvinyl chloride with silicone rubber tips and advanced to the abdominal aorta and vena cava via the left femoral artery and vein in rats anesthetized with pentobarbital (50 mg/kg i.p.). The ends of the catheters were tunneled s.c. to the head, where the catheters were stabilized to the skull using jeweler’s screws and dental acrylic. Catheter ends were passed through a stainless steel spring attached to a plastic swivel, through which infusions were given. Upon regaining consciousness, rats were housed singly in stainless steel cages in a climate-controlled room infusions were given. Upon regaining consciousness, rats were housed singly in stainless steel cages in a climate-controlled room with a 12-h light/dark cycle. At least 1 week of recovery was allowed before experiments were begun.

(+)–Norfenfluramine (10–300 μg/kg i.v.) was given in a cumulative fashion in 6-min intervals. Mean arterial blood pressure and heart rate were monitored prior to, during, and for 6 min after each injection with a computerized DigitMed system (DigitMed Systems, Madrid, Spain). Ketanserin was given 30 min before (+)–norfenfluramine.

Statistics
Contractile data are expressed as mean ± S.E.M. and reported as a percentage of the maximal contraction to PE (10 μM/KCl) (67 mM). Unpaired Student’s t tests were performed, and p ≤ 0.05 was considered statistically significant. Agonist EC50 values were calculated using a nonlinear regression analysis using the algorithm [effect = maximal response/1 + (EC50/agonist concentration)] in the program Prism (GraphPad Software Inc., San Diego, CA). Apparent antagonist dissociation constants (Kd) values were calculating using eq. 1:

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\log (d - 1) = \log [B] - \log K_d
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where d is the EC50 value of agonist in the presence of the antagonist divided by the EC50 value of agonist in the absence of the antagonist, and [B] is the concentration of the antagonist tested. Blood pressure is reported as a change of mean arterial blood pressure.

Chemicals
Acetylcholine chloride, deoxycorticosterone acetate, 5-hydroxytryptamine hydrochloride, ketanserin tartrate, phenylephrine hydrochloride, and LY53857 were purchased from Sigma Chemical Co. (St. Louis, MO). LY272015 was a generous gift from Eli Lilly and Company (Indianapolis, IN), and (+)–norfenfluramine was generously provided by SRI International (Menlo Park, CA).

Results
Response of Arteries from Normotensive and Hypertensive Rats to (+)-Norfenfluramine. SHAM rats had a systolic blood pressure of 117 ± 4 mm Hg and DOCA-salt, 185 ± 7 mm Hg. Figure 1 compares responses of thoracic aorta (Fig. 1a), renal artery (Fig. 1b), and mesenteric resistance artery (Fig. 1c) from normotensive and DOCA-salt hypertensive rats with (+)-norfenfluramine. (+)-Norfenfluramine contracted arteries in a concentration-dependent manner with a dramatic decrease in threshold (aorta and mesenteric resistance artery) (Table 1; Fig. 1, a and c), increase in potency (EC50 value decreased, all arteries), and increase in maximal response (aorta and mesenteric resistance artery) in arteries from DOCA-salt rats compared with arteries from SHAM rats (Table 1). These data are reported as a percentage of an initial PE (10 μM) contraction (Table 1); PE contraction in terms of maximal response and EC50 values was not significantly different (p > 0.05, Student’s t test) between arteries from SHAM and DOCA rats (data not shown).

Existence of the 5-HT2A Receptor in Aorta from SHAM and DOCA-Salt Rats. Immunohistochemical experiments used an antibody that recognized the N-terminal synthetic sequence corresponding to amino acids 22 to 41 of rat 5-HT2A receptor and localized the 5-HT2A receptor protein to endothelial and smooth muscle layers of the aorta section from both SHAM and DOCA-salt rats (Fig. 2a, left, arrows). A lighter staining was observed in aorta incubated with primary antibody plus competing peptide (middle), suggesting specific 5-HT2A receptor localization. The staining on adventitia was competed off by competing peptide in aorta from SHAM rats but not DOCA-salt rats. The right picture shows nonprimary control. All slides were counterstained with Vector hematoxylin to indicate the nuclei (blue staining) of smooth muscle cells lying between bundles of collagen and elastin.

Figure 2b depicts ethidium bromide-stained products of real-time RT-PCR for 5-HT2A receptor mRNA. We observed one product of the expected size (141 bp) in all of aorta samples from SHAM and DOCA rats. β-Actin was used as a control, Ct values were measured as the value at which measurable product was first observed in real-time RT-PCR. We did not detect a difference in 5-HT2A receptor mRNA expression in aorta samples from SHAM and DOCA rats as the ∆Ct values of 5-HT2A receptor mRNA were not significantly different (5-HT2A receptor ∆Ct – β-actin ∆Ct), SHAM = 12.23 ± 0.50, DOCA = 11.82 ± 0.33; p = 0.09; n = 4–5).

Effects of 5-HT Receptor Antagonists on (+)-Norfenfluramine-Induced Contraction in Aorta from SHAM and DOCA-Salt Hypertensive Rats. To determine the receptor type mediating (+)-norfenfluramine-induced enhanced contraction in DOCA-salt rats, contraction in aorta was examined in the presence of 5-HT2A receptor antagonist ketanserin and 5-HT2A antagonist receptor antagonist LY53857. Ketanserin (Fig. 3a; 10 nM) competitively shifted the (+)-norfenfluramine concentration-response curve rightward 29.5-fold in aorta from normotensive rats and 20.4-fold in DOCA-salt rats. The apparent dissociation constant calculated from these shifts were 9.54 ± 0.21 in SHAM rats and estimated as 9.25 ± 0.21 in DOCA rats. LY53857 inhibited (+)-norfenfluramine-induced contraction in aorta from
SHAM rats (-Log EC50, vehicle = 5.54 ± 0.13; LY53857, 3.32 ± 0.17) and DOCA-salt rats (-Log EC50, vehicle = 6.23 ± 0.09; LY53857, 4.93 ± 0.24) (Fig. 3b). (+)-Norfenfluramine-induced rat stomach fundus contraction was inhibited by 10 nM 5-HT2A/2B receptor antagonist, LY272015. We then tested the effect of the same concentration of LY272015 on (+)-norfenfluramine-induced aortic contraction in SHAM and DOCA-salt hypertensive rats. LY272015 (Fig. 3d) did not shift (+)-norfenfluramine-induced contraction in aorta from SHAM or DOCA-salt rats.

Response to (+)-Norfenfluramine in Whole Animal.
We reported (+)-norfenfluramine increased blood pressure in a dose-dependent and 5-HT2A receptor-dependent manner in conscious normal rats with no concomitant change in heart rate (Ni et al., 2004a). In this study, we compared the (+)-norfenfluramine pressure effect in conscious SHAM and DOCA-salt rats (basal mean arterial blood pressure, SHAM = 104 ± 5 mm Hg; DOCA = 145 ± 7 mm Hg). Figure 4 depicts effects of ketanserin on the (+)-norfenfluramine-induced pressor response in conscious SHAM and DOCA-salt rats (change in mean arterial blood pressure at 300 μg/kg, mm Hg, SHAM vehicle = 36 ± 4, SHAM ketanserin = 7 ± 2, DOCA = 51 ± 5, DOCA ketanserin = 19 ± 1). The pressor effect of (+)-norfenfluramine was significantly increased in DOCA-salt hypertension compared with SHAM rats. The pressor response to (+)-norfenfluramine in SHAM and DOCA rats was inhibited by the 5-HT2A receptor antagonist (ketanserin; Fig. 4).

Discussion
Our results showed that effects of (+)-norfenfluramine both in vitro and in vivo were significantly enhanced in mineralocorticoid hypertension, and these effects are dependent on activation of the 5-HT2A receptor.

Enhanced Arterial Contraction to (+)-Norfenfluramine. (+)-Norfenfluramine caused a concentration-dependent contraction in multiple arteries from SHAM and DOCA-
salt hypertensive rats (Fig. 1, a–c). We observed enhanced (+)-norfenfluramine-induced contraction in both conduit (aorta, renal artery) and resistance arteries (mesenteric resistance artery) (Fig. 1, a–c); thus, potentiated response to (+)-norfenfluramine in DOCA-salt rats arteries is not vessel-specific. In the following experiments, we used aorta as a model to investigate the role of 5-HT receptors in (+)-norfenfluramine-induced contraction in DOCA-salt hypertension.

To investigate whether (+)-norfenfluramine-induced contraction in arteries from DOCA-salt rats depends on the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors, we first tested for the existence of the 5-HT$_{2A}$ receptor in aorta. The up-regulated expression and function of 5-HT$_{2B}$ receptors (Watts et al., 1996; Banes and Watts, 2002) in aorta from DOCA-salt rats has been reported. However, without the use of a specific 5-HT$_{2A}$ receptor agonist, it has been difficult to prove this receptor is still present and functional in arteries of hypertensive animals. Our immunohistochemistry experiment used an antibody that recognizes the 5-HT$_{2A}$ receptor and localized this receptor to endothelium, smooth muscle, and possibly adventitial layers, confirmed by using competing peptide and no primary negative control (Fig. 2a). Although the staining of 5-HT$_{2A}$ receptor is lighter in aorta from DOCA-salt rats compared with SHAM, it is not proper to draw the conclusion that less 5-HT$_{2A}$ receptor expressed by the pictures from immunohistochemistry experiments. These pictures are intended to demonstrate qualitatively the existence of the 5-HT$_{2A}$ receptor in arteries of DOCA-salt rats. We tried Western analysis to compare the amount of 5-HT$_{2A}$ receptor expressed in aorta from SHAM and DOCA rats. Unfortunately, the many 5-HT$_{2A}$ receptor antibodies available are not useful in quantifying the 5-HT$_{2A}$ receptor protein with this technique. Due to this reason, we performed a real-time RT-PCR to test the expression of 5-HT$_{2A}$ receptor mRNA. Our results showed an expression of 5-HT$_{2A}$ receptor mRNA in aorta from SHAM and DOCA rats, suggesting the possibility of a local 5-HT$_{2A}$ receptor protein synthesis. The expression levels of mRNA were similar in aorta from SHAM compared with that in DOCA rats, which may or may not indicate a similar amount of 5-HT$_{2A}$ receptor protein expression because there could be post-transcriptional regulations.

We then examined the pharmacology of (+)-norfenfluramine with 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor antagonists in arteries from DOCA-salt hypertensive rats. We have demon-
from normal rats. d, effect of 5-HT2B receptor antagonist LY272015 (10 nM) on (+)-norfenfluramine-induced contraction in fundus/H11001/H11001-norfenfluramine-induced aortic contraction in aorta from normotensive and hypertensive rats. c, effect of 5-HT2A/2B receptor antagonist LY53857 (30 nM) on (+)-norfenfluramine-induced contraction in aorta from normotensive and hypertensive rats. Points represent means, and vertical bars represent the S.E.M. for the number of animals indicated in parentheses. *, statistically significant differences from SHAM rats (p < 0.05); #, statistically significant differences from control response (vehicle) (p < 0.05).

Fig. 3. a, effect of 5-HT2A receptor antagonist ketanserin (10 nM) on (+)-norfenfluramine-induced contraction in aorta from normotensive and hypertensive rats. b, effect of 5-HT2A/2B receptor antagonist LY53857 (30 nM) on (+)-norfenfluramine-induced contraction in aorta from normotensive and hypertensive rats. c, effect of 5-HT2B receptor antagonist LY272015 (10 nM) on (+)-norfenfluramine-induced contraction in aorta from normotensive and hypertensive rats. d, effect of 5-HT2B receptor antagonist LY272015 (10 nM) on (+)-norfenfluramine-induced contraction in aorta from normotensive and hypertensive rats. Points represent means, and vertical bars represent the S.E.M. for the number of animals indicated in parentheses.

Nevertheless, in this study, we showed that the 5-HT2A receptor was still involved in (+)-norfenfluramine-induced aortic contraction in DOCA-salt hypertension. This is important because it has not yet been proven that the 5-HT2A receptor is functional in arteries from DOCA-salt rats. The response to low concentrations of 5-HT (1–300 nM) was not inhibited by ketanserin (30 nM) in aorta (Watts, 2002) and superior mesenteric artery (Watts et al., 1996) from DOCA-salt rats. Arterial contraction to low concentrations of 5-HT is likely due to 5-HT2B receptor activation. Thus, the lack of ability of ketanserin to block 5-HT-induced contraction in aorta from DOCA-salt rats could be because of dysfunction of the 5-HT2A receptor or masking by 5-HT2B receptor activation. Here, we performed experiments suggesting that the 5-HT2A receptor protein was preserved and functional in aorta from DOCA-salt rats.

(+)-Norfenfluramine can function as a 5-HT2B receptor agonist because we observed a reduced (+)-norfenfluramine-induced contraction in rat stomach fundus by the 5-HT2B receptor antagonist LY272015 (10 nM; Fig. 3c). Previous studies showed that the 5-HT2B receptor was up-regulated in DOCA-salt rats (Watts et al., 1996; Banes and Watts, 2002; Watts, 2002) because we observed that the 5-HT2B receptor antagonist LY272015 reduced 5-HT-induced contraction in aorta from and blood pressure (Watts and Fink, 1999) in DOCA rats but not SHAM rats. We expected to observe that 5-HT2B receptors played a role in (+)-norfenfluramine-induced contraction in vessels from DOCA-salt rats. Contrary to our hypothesis, we did not observe inhibition of (+)-norfenfluramine-induced contraction in DOCA-salt hypertensive rats by the selective 5-HT2B receptor antagonist LY272015. Then, we tried using higher concentrations of LY272015 (30 and 50 nM). At these concentrations, LY272015 inhibited (+)-norfenfluramine-induced contraction in aorta from both SHAM and DOCA rats (data not shown). The affinities of LY272015 at the human 5-HT2A and
5-HT$_{2B}$ receptors have been reported as: 5-HT$_{2A}$, 28.7 ± 2.3 nM; and 5-HT$_{2B}$, 0.75 ± 0.06 nM (Cohen et al., 1996). Thus, we do not believe that LY272015 at these higher concentrations (30 and 50 nM) exert functions specifically to inhibit 5-HT$_{2B}$ receptors.

It is possible that the abundance of the 5-HT$_{2B}$ receptor, even though increased in DOCA-salt rats, is not sufficient for (+)-norfenfluramine to induce contraction. Thus, the data do not support the idea that 5-HT$_{2B}$ receptors play a role in (+)-norfenfluramine-induced contraction in arteries from SHAM or DOCA-salt hypertensive rats.

(+)-Norfenfluramine as a Pressor Agent. (+)-Fenfluramine has been reported to increase blood pressure in many studies (Mabadeje, 1974; Michelakis et al., 1999) because the active metabolite of (+)-fenfluramine, (+)-norfenfluramine, has a significantly longer half-life (20 versus 32 h) (http://www.ispub.com/ostia/index.php?xmlFilePath=ijanj/vol11/dietpill.xml). Thus, it is logical to speculate that the (+)-fenfluramine pressor responses in the above reports were caused, at least partially, by (+)-norfenfluramine. More importantly, we reported previously that (+)-norfenfluramine (10–300 µg/kg i.v.) caused a dose-dependent increase in blood pressure in conscious normal rats without a concomitant change in heart rate (data not shown) via activation of the 5-HT$_{2A}$ receptor (Ni et al., 2004b). In this study, we observed an increased pressor effect of (+)-norfenfluramine in hypertensive animals, which is consistent with the increased contractile response to (+)-norfenfluramine in arteries from DOCA-salt hypertensive rats. Thus, it is possible that increased sensitivity to (+)-norfenfluramine in systemic arteries (Fig. 1, a–c) leads to increased peripheral resistance and blood pressure. Although ketanserin (3 mg/kg) reduced (+)-norfenfluramine-induced pressor responses to a similar extent in SHAM and DOCA-salt rats, ketanserin abolished blood pressure increases in SHAM but not in DOCA-salt rats. This is consistent with our in vitro observation of less rightward shift of (+)-norfenfluramine concentration response curve by ketanserin in aorta from DOCA-salt rats (Fig. 3).

In our in vivo and in vitro data indicated that in SHAM rats, the 5-HT$_{2A}$ receptor is the primary receptor responsible for (+)-norfenfluramine-induced vasoconstriction and blood pressure increase, whereas in DOCA-salt rats, in addition to 5-HT$_{2A}$ receptor, other receptors or mechanisms are also involved in these effects.

In summary, our study suggests that (+)-fenfluramine or (+)-norfenfluramine as a weight control drug might have a greater peripheral artery vasoconstriction and pressor effect in individuals who already have high blood pressure. We also proved that the 5-HT$_{2A}$ receptors are preserved and functional in arteries from DOCA-salt hypertensive rats. As a 5-HT$_{2A}$ receptor agonist without activating arterial 5-HT$_{2B}$ receptor, (+)-norfenfluramine might be a useful tool for serotonin research in peripheral vasculature.

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