(+)-Norfenfluramine-Induced Arterial Contraction Is Not Dependent on Endogenous 5-Hydroxytryptamine or 5-Hydroxytryptamine Transporter

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ABSTRACT

(+)-Norfenfluramine, the major metabolite of fenfluramine, causes vasoconstriction dependence on the 5-hydroxytryptamine (5-HT)2A receptor in rat. (+)-Norfenfluramine was reported as a 5-hydroxytryptamine transporter (5-HTT) substrate and 5-HT releaser. Because the arterial 5-HTT exists and is functional in the rat, we hypothesized that (+)-norfenfluramine causes vasoconstriction by releasing 5-HT from vascular smooth muscle via 5-HTT. The released 5-HT, in turn, activates the 5-HTT2A receptor. Isometric contractility experiments showed that (+)-norfenfluramine-induced mouse aortic contraction was reduced by the 5-HTT inhibitor fluoxetine (1 μM) but not by fluvoxamine (1 μM). Tryptophan hydroxylase (TPH)-deficient (Tph1−/−) mice lack peripheral 5-HT. (+)-Norfenfluramine (10 nM–100 μM)-contracted aorta from wild-type and Tph1−/− mice with equivalent potency (−log EC50 [M], wild type = 5.73 ± 0.02, Tph1−/− = 5.62 ± 0.09), and these contractions were inhibited by the 5-HTT2A receptor antagonist ketanserin (3 nM) by a similar magnitude in aorta from wild-type and Tph1−/− mice (wild type = 19.4, Tph1−/− = 15.4-fold rightward shift versus control), as did fluoxetine (1 μM) (wild type = 22.4, Tph1−/− = 28.8-fold rightward shift versus control). To further test the role of 5-HTT in (+)-norfenfluramine-induced aortic contraction, the 5-HTT-targeted mutation mouse was used. (+)-Norfenfluramine induced similar aortic constriction in wild-type and 5-HTT-targeted mutation mice, and these contractions were inhibited by fluoxetine (1 μM). Thus, (+)-norfenfluramine vasoconstriction is not dependent on 5-HTT-mediated release of endogenous 5-HT but by activating membrane 5-HTT2A receptors directly. Understanding of the mechanism by which (+)-norfenfluramine induces vasoconstriction is important to characterize and understand the function of the serotonergic system in peripheral arterial vasculature.

The highly effective anorexigen (+)-fenfluramine (Redux) was widely prescribed for the treatment of obesity until it was associated with primary pulmonary hypertension (Abenhaim et al., 1996) and aortic valvular disease (Connolly et al., 1997). We have reported previously that the hepatic deethylated metabolite of (+)-fenfluramine, (+)-norfenfluramine, is vasoactive. (+)-Norfenfluramine causes constriction in isolated rat aorta, renal artery, and mesenteric resistance artery and increases blood pressure through activation of 5-hydroxytryptamine (5-HT)2A receptor (Ni et al., 2004a).

Recently, we found that 5-HT and a functional 5-HTT are present in rat peripheral arteries (Ni et al., 2004b). 5-HT is a vasoconstrictor and causes constriction in many arteries and veins, especially conduit vessels via 5-HTT2A receptors (Martin, 1994). (+)-Fenfluramine and (+)-norfenfluramine are 5-HTT substrates and potent 5-HT releasers (Garattini, 1995; Rothman and Baumann, 2002). Because the anorexic effect of (+)-fenfluramine was considered to be due at least in part to 5-HT release (Fishman, 1999), we hypothesized that (+)-norfenfluramine-induced vasoconstriction is dependent on release of endogenous 5-HT via 5-HTT with consequent 5-HTT2A receptor-mediated contraction.

We studied our hypothesis by using two different strains of mice. Aorta from tryptophan hydroxylase (TPH) 1-deficient...
(Tph1−/−) mice, which lack 5-HT synthesis in the periphery but have normal level of 5-HT in brain, were used to investigate the role of endogenous arterial 5-HT in (+)-norfenfluramine-induced arterial contraction. The 5-HTT-targeted mutation mouse lacks a functional 5-HTT (Bengel et al., 1998) and is insensitive to blockade by the 5-HTT inhibitor fluoxetine (Holmes et al., 2002). We used this mouse model to demonstrate the role of 5-HTT in (+)-norfenfluramine-induced contraction.

In this study, we first provided evidence for the presence of 5-HT and a functional 5-HTT in mouse aorta and investigated the dependence of (+)-norfenfluramine-induced contraction on 5-HTT by using 5-HTT inhibitors fluoxetine and fluvoxamine. Then, we investigated whether (+)-norfenfluramine-induced contraction was dependent on endogenous 5-HT using Tph1−/− mice and the role of 5-HTT in (+)-norfenfluramine-induced contraction by using 5-HTT-targeted mutation mice. Last, we studied the different effects of fluoxetine and fluvoxamine on 5-HT uptake in mouse aorta.

Materials and Methods

All procedures that involved animals were performed in accordance with the institutional guidelines of Michigan State University.

Animal Use

The normal male C57BL/6 mice (20–24 g; Charles River Breeding Laboratories, Portage, MI), Tph1−/− mice and wild-type male mice (C57BL/6; 20–24 g; Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany), 5-HTT-targeted mutation mice and wild-type male mice (C57BL/6; 30–40 g; National Institute of Mental Health, Bethesda, MD) were used in the experiments.

High-Pressure Liquid Chromatography (HPLC) Isolation of 5-HT and 5-Hydroxyindole Acetic Acid (5-HIAA)

Aortas from vehicle or pargyline (100 mg/kg i.p.; 30 min)-treated mice were dissected, cleaned, and placed in tissue buffer (0.05 mM sodium phosphate and 0.03 mM citric acid buffer, pH 2.5, containing 15% methanol). Samples were frozen in −80°C until assay. Samples were thawed, sonicated for 3 s, and centrifuged for 30 s (10,000 g). Supernatant was collected and transferred to new tubes. Tissue pellets were dissolved in 1.0 M NaOH and assayed for protein. Concentrations of 5-HIAA and 5-HT in tissue supernatants were determined by isocratic HPLC coupled with electrochemical detection (Chapin et al., 1986).

Western Analysis

Protein Isolation. Mouse aorta were cleaned, pulverized in liquid nitrogen, and solubilized in lysis buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, and 10% glycerol) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Homogenates were centrifuged (11,000g for 10 min; 4°C), and supernatant total protein was measured.

Immunoprecipitation. Mouse aorta protein homogenates (200 μg) were incubated with 2 μg/ml 5-HTT C-20 antibody (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) for 2 h. Twenty microliters of protein A/G-agarose was added and tumbled overnight at 4°C. Beads were collected by centrifugation at 2500 rpm for 30 s, and supernatant was discarded. Beads were washed three times with phosphate-buffered saline (PBS), each time repeating the centrifugation step mentioned above. After the final wash, the beads were boiled in 40 μl of 2% lysis buffer and centrifuged, and the supernatant was loaded on SDS polyacrylamide gel for Western analysis.

Western Blotting. Fifty micrograms of total protein or 10 μl of immunoprecipitated samples was separated on 10% SDS polyacrylamide gels using a Mini Bio-Rad III apparatus. Membranes were blocked for 3 h in 4% chick egg ovalbumin (4°C Tris-buffered saline (TBS)-0.1% Tween + 0.025% NaN3). Primary antibody (0.5 μg/ml, 5-HTT C-20; Santa Cruz Biotechnology) was incubated with blots overnight at 4°C. Blots were then rinsed three times in TBS-0.1% Tween with a final rinse in TBS and incubated with donkey peroxidase-linked anti-goat secondary antibody (1:2000; Santa Cruz Biotechnology) for 1 h at 4°C with rocking. ECL reagents (Amersham Biosciences, Inc.; Piscataway, NJ) were used to visualize bands.

Immunohistochemistry

Mouse aorta was snap frozen in Tissue-Tek optimal cutting temperature (O.C.T.) compound and stored at −70°C until use. Arterial sections (8 μm) were cut and air-dried (overnight at room temperature). Samples were cold (4°C) acetone-fixed, washed three times with PBS, and endogenous peroxidase-blocked (0.3% H2O2 in PBS for 30 min). Sections were blocked for nonspecific binding in PBS containing 1.5% of competing serum. In a humidified chamber, samples incubated 24 h with antibody (5-HTT C-20; Santa Cruz Biotechnology, Inc.; 4°C, 5 μg/ml with 1.5% blocking serum in PBS) or antibody neutralized with 5-fold excess of competing peptide. The remaining steps were carried out in accordance to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). Sections were washed three times with PBS and incubated with a peroxidase-conjugated secondary antibody (30 min at room temperature). Samples were washed and incubated with Vectastain ABC Elite reagent (30 min at room temperature) followed by 3,3′-diaminobenzidine/H2O2. Reaction was stopped with washing, sections air-dried, hematoxylin-stained, mounted, and photographed using a Spot 2 digital camera on a Leica light microscope with filters.

Isolated Tissue Bath Assay

Thoracic aorta from wild-type mice, Tph1−/− mice, and 5-HTT-targeted mutation mice were removed and placed in physiological salt solution (PSS). Vessels were trimmed of fat and cut into helical strips (0.15 × 0.75 cm). 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 (103 mM NaCl, 4.7 mM KCl, 1.18 mM KH2PO4, 1.17 mM MgSO4·7H2O, 1.6 mM CaCl2·2H2O, 14.9 mM NaHCO3, 5.5 mM dextrose, and 0.03 mM CaNa2-EDTA), warmed to 37°C and aerated with 95% oxygen and 5% carbon dioxide. Each strip was placed under optimum resting tension (previously determined, 250 mg; Russell and Watts, 2000) and allowed to equilibrate for 1 h with frequent buffer changes. Tissues were then challenged with a maximal concentration of the a1-adrenergic agonist phenylephrine (10−5 M) 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 phenylephrine (10−6–10−7 M), and once the contraction plateaued, the muscarinic agonist acetylcholine (10−5 M) was administered. A relaxation to acetylcholine greater than 60% of the phenylephrine-induced contraction in those tissues in which endothelium was intact. Tissues were again washed until baseline was reached, and then one of the following protocols was used.

Protocol 1. Testing of Response to Agonists: 5-HT and (+)-Norfenfluramine. Concentration-response curves to 5-HT and (+)-norfenfluramine were performed in a cumulative manner. Each concentration incubated a minimum of 3 min. When contraction reached a maximum, the next higher concentration of agonist was added. Contraction to agonist was generated.

Protocol 2. Testing of Effect of Antagonist or Inhibitor on (+)-Norfenfluramine-Induced Contraction. Vehicle or antagonist/inhibitor was added to the bath for 1 h. At this time, a cumulative response to (+)-norfenfluramine in the presence of vehicle or antagonist was generated.
5-HT Uptake Assay

At room temperature, dissected and washed aorta were placed in 5-HT (1 μM, diluted in PSS) or vehicle (water + PSS) in 1.5-ml plastic centrifuge tubes for 15 min. Tissues were then briefly dipped in drug-free PSS and placed in 75 μl of tissue buffer. Samples were frozen (−80°C) until assay. Tissues were incubated with vehicle or 5-HTT inhibitor 30 min before exposure to exogenous 5-HT when testing the effect of 5-HTT inhibitor.

Data Analysis

5-HIAA and 5-HT concentration detected by HPLC was quantified using standards run the same day and reported as a concentration relative to protein content or as percentage of vehicle group in 5-HT uptake experiments. Band density quantitation in Western analysis was performed using NIH Image (version 1.61). When comparing two groups, unpaired Student’s t tests were used because control and treated samples were different tissues. Contractile data are expressed as ± S.E.M. and are reported as a percentage of the maximal contraction to phenylephrine (10−5 M). Unpaired t tests were performed and a p value ≤0.05 was considered statistically significant. Agonist EC50 values were calculated using a nonlinear regression analysis using the algorithm [effect = maximum response/1+(EC50/ agonist concentration)] in the program GraphPad Prism (GraphPad Software Inc., San Diego, CA). Apparent antagonist dissociation constants (Kd values) were calculating using the following equation: log (dr−1) = log [B]−log Kd, where dr 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.

Chemicals

Acetylcholine chloride, 5-hydroxytryptamine hydrochloride, ketanserin tartrate, fluoxetine hydrochloride, fluvoxamine maleate, pargyline hydrochloride, and phenylephrine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). (+)-Norfenfluramine was graciously provided by SRI International (Menlo Park, CA).

Results

Measurement of Basal Level of 5-HT and 5-HIAA in Normal Mouse Aorta. Most studies measuring 5-HT uptake use [3H]5-HT, which only measures 5-HT but not its metabolite. We used HPLC-based measurements of arterial 5-HT and 5-HIAA on an HPLC system that allowed concurrent measurement of amines in the same samples. We measured basal level of 5-HT (1.39 ± 0.24 ng/mg protein) and the monoamine oxidase-A metabolite 5-HIAA (0.27 ± 0.04 ng/mg protein) in normal C57BL6 mouse aorta (Fig. 1A). 5-HIAA production was abolished and 5-HT concentration was increased (no significance) in aorta from pargyline-treated mice (5-HT = 2.03 ± 0.38 ng/mg protein; 5-HIAA = 0.015 ± 0.009 ng/mg protein, Fig. 1A). These data suggest that basal level of 5-HT and 5-HIAA are detectable and exist in a metabolically active location in mouse aorta.

Presence of 5-HT and 5-HTT in Mouse Aorta. Western analysis using an antibody specific for recognizing 5-HTT C terminus (C-20; Santa Cruz Biotechnology, Inc.) was performed in mouse aorta whole tissue homogenate supernatant (Fig. 1B, homog.) and immunoprecipitated protein (Fig. 1B, IP). These bands in both straight homogenate or immunoprecipitated protein samples migrated at ~70 kDa, consistent with that reported for brain and lung 5-HTT (between 60 and 80 kDa).

Immunohistochemical experiments using the same antibody localized the 5-HTT protein to smooth muscle and the endothelial layer of the mouse aorta (Fig. 1C, compare left and middle). The black staining in adventitia was not competed off by competing peptide; thus, this staining is likely nonspecific. The right picture shows a mouse aorta section incubated with secondary antibody but no primary antibody.
The blue staining is the nuclei of smooth muscle cells lying between bundles of collagen and elastin.

**(+)-Norfenfluramine-Induced Aortic Constriction and Dependence on 5-HTT.** Figure 2 shows the concentration-response curve to (+)-norfenfluramine in aorta from C57BL/6 mice. Similar to that observed in rat aorta (Ni et al., 2004a), (+)-norfenfluramine contracted the mouse aorta in a concentration-dependent manner with a −log EC50 value [M] of 5.68 ± 0.03. To determine whether 5-HTT plays a role in (+)-norfenfluramine-induced vasoconstriction, contraction in aorta was examined in the presence of the 5-HTT inhibitors fluoxetine (1 µM) or fluvoxamine (1 µM). The (+)-norfenfluramine-induced contraction was shifted 22-fold rightward (−log EC50 value [M] = 4.39 ± 0.04, pKᵦ value = 7.29 ± 0.06) by fluoxetine (1 µM). However, a different 5-HTT inhibitor, fluvoxamine, showed no effect on (+)-norfenfluramine-induced aortic contraction (−log EC50 value [M] = 5.75 ± 0.17). These data can be interpreted to mean that either (+)-norfenfluramine-induced contraction is dependent on 5-HTT function through 5-HT release and/or fluoxetine has inhibited receptors mediating (+)-norfenfluramine-induced contraction.

**Measurement of Basal Level 5-HT/5-HIAA in Tph1−/− Mouse Aorta and 5-HT Uptake Ability in Wild-Type and Tph1−/− Mouse Aorta.** To examine the dependence of endogenous 5-HT in (+)-norfenfluramine-induced vasoconstriction, we used Tph1−/− mice. Tph1−/− mice express normal amounts of 5-HT in brain but lack 5-HT in the periphery (Walther et al., 2003a). Figure 3 shows 5-HIAA and 5-HT concentrations in aorta from Tph1−/− mice with vehicle or with exogenous 5-HT (1 µM; 15 min). As expected, aorta from Tph1−/− mice had significantly reduced 5-HT (0.083 ± 0.049 ng/mg protein) and 5-HIAA (0.023 ± 0.023 ng/mg protein) concentrations compared with wild-type mice (5-HT = 0.49 ± 0.11 ng/mg protein; 5-HIAA = 0.37 ± 0.17 ng/mg protein).

To ensure the 5-HTT in mouse aorta was functional, we incubated wild-type and Tph1−/− mice with 5-HT (1 µM) for 15 min. The (−)-norfenfluramine-induced contraction in aorta from wild-type and Tph1−/− mice showed similar ability to take up exogenous 5-HT. After incubation with exogenous 5-HT (1 µM) for 15 min, the concentration of 5-HT and 5-HIAA increased in aorta from C57BL/6 wild-type mouse (5-HT = 0.85 ± 0.19 ng/mg protein; 5-HIAA = 1.75 ± 0.25 ng/mg protein; Fig. 3) and Tph1−/− mouse (5-HT = 0.62 ± 0.13 ng/mg protein; 5-HIAA = 1.54 ± 0.40 ng/mg protein). This indicates that the 5-HTT in wild-type and Tph1−/− mice has normal function.

**(+)-Norfenfluramine-Induced Aortic Constriction and Dependence on Endogenous 5-HT.** Using the Tph1−/− mice, we next performed a cumulative 5-HT concentration-response curve. 5-HT caused a concentration-dependent contraction in aorta from Tph1−/− mice (−log EC50 value [M] = 6.90 ± 0.03) that was similar with that in wild-type mice (−log EC50 value [M] = 6.96 ± 0.03) (Fig. 4A). Thus, aortas from Tph1−/− mice have a normal contractile response to 5-HT.

Contrary to our hypothesis, (+)-norfenfluramine caused a concentration-dependent contraction in aorta from Tph1−/− mice with a −log EC50 value [M] = 5.62 ± 0.09 (Fig. 4B and C). The potency of (+)-norfenfluramine-induced contractions was similar in aorta from wild-type and Tph1−/− mice (−log EC50 value [M] = 5.74 ± 0.02). These data suggest that (+)-norfenfluramine-induced contraction is independent of endogenous 5-HT release.

**Effect of 5-HT2A Receptor Antagonist Ketanserin and 5-HTT Inhibitor Fluoxetine on (+)-Norfenfluramine-Induced Contraction in Aorta from Tph1−/− Mice.** To determine the role of 5-HT2A receptor and 5-HTT in (+)-norfenfluramine-induced contraction, mouse aortas were examined in the presence of 5-HT2A receptor antagonist ketanserin or 5-HTT inhibitor fluoxetine. Ketanserin (3 nM) competitively shifted the (+)-norfenfluramine concentration-response curve rightward 19-fold in aorta from wild-type mice (Fig. 4B). The apparent dissociation constant calculated from this shift (pKᵦ = 9.79 ± 0.13) is consistent with antagonism of the 5-HT2A receptor.

We also tested the effect of ketanserin and fluoxetine on (+)-norfenfluramine-induced contraction in aorta from Tph1−/− mice. Ketanserin inhibited (+)-norfenfluramine-
induced contraction, with a $pK_a$ value $= 9.67 \pm 0.17$ (Fig. 4B), similar to the $pK_a$ value from wild-type animal and consistent with inhibition of the 5-HT$_{2A}$ receptor. Fluoxetine also rightward shifted (+)-norfenfluramine response curve (29-fold, $-\log EC_{50}$ value [M] = 4.15 \pm 0.07, $pK_a$ value = 7.27 \pm 0.19; Fig. 4C) in aorta from Tph1$^{-/-}$ mice aorta, similar to what we observed in wild-type mice paired with Tph1$^{-/-}$ and C57BL/6 mice from Charles River Breeding Laboratories (Fig. 2). Thus, the 5-HT$_{2A}$ receptor and potentially 5-HTT but not 5-HT release played important roles in (+)-norfenfluramine-induced contraction in aorta from Tph1$^{-/-}$ mice.

Measurement of (+)-Norfenfluramine and 5-HT-Induced Contraction in 5-HTT-Targeted Mutation Mouse (5-HTT KO) and the Effect of Fluoxetine. We next used 5-HTT-targeted mutation mice in our study to test the dependence of (+)-norfenfluramine-induced contraction on 5-HTT and to determine whether effects of fluoxetine are 5-HTT-dependent. In aorta isolated from wild-type and 5-HTT KO mice, cumulative (+)-norfenfluramine concentration-response curves were generated in the presence of vehicle or fluoxetine (1 \mu M). (+)-Norfenfluramine induced a concentration-dependent contraction in both wild-type and 5-HTT KO mice ($-\log EC_{50}$ [M], wild type = 5.54 \pm 0.03, 5-HTT KO = 5.52 \pm 0.14; Fig. 5A). Fluoxetine inhibited aortic contraction to (+)-norfenfluramine in wild-type (18.7-fold rightward shift), and, by a similar magnitude, in 5-HTT KO mouse aorta (17.4-fold rightward shift). Figure 5B shows 5-HTT-induced concentration-dependent mouse aortic contraction ($-\log EC_{50}$ [M], wild type = 6.93 \pm 0.11, 5-HTT KO = 6.99 \pm 0.12) was inhibited by fluoxetine (1 \mu M) in wild-type and 5-HTT KO ($-\log EC_{50}$ [M], wild type = 5.63 \pm 0.10, 5-HTT KO = 5.46 \pm 0.32). Therefore, (+)-norfenfluramine-induced contraction was not dependent on 5-HTT, and fluoxetine may have pharmacological effects additional to 5-HTT blockade.

Effect of 5-HTT Inhibitors Fluoxetine and Fluvoxamine on 5-HT Uptake in Mouse Aorta. To investigate the different effect of fluoxetine and fluvoxamine on mouse aorta, we compared the effect of these two 5-HTT inhibitors on 5-HT uptake in mouse aorta. Figure 6 shows 5-HT concentrations in each treatment group as percentage of control group in a 5-HT uptake study using aorta from pargyline-treated C57BL6 mice. Pargyline was used in this experiment to inhibit monoamine oxidase-A and thus inhibited 5-HT metabolite to 5-HIAA. Aortic 5-HIAA concentrations were almost abolished after treated with pargyline; thus, we reported and only compared 5-HT concentrations. Fluoxetine (1 \mu M) on (+)-norfenfluramine-induced cumulative contraction in aorta from wild-type mice and 5-HTT-targeted mutation mice. Points represent means and vertical bars the S.E.M. for the number of animals indicated in parentheses. *, statistical difference ($p < 0.05$) versus control in wild-type mice. #, statistical difference ($p < 0.05$) versus vehicle in wild-type mice.
µM) alone moderately increased 5-HT concentrations (not significantly different from control) and did not inhibit 5-HT uptake in mouse aorta (Fig. 6A). By contrast, fluvoxamine (1 µM) had no effect on basal level 5-HT and significantly reduced 5-HT uptake in mouse aorta (Fig. 6B). Thus, fluoxetine and fluvoxamine had different effects on 5-HT uptake in mouse aorta.

Discussion

(+)-Fenfluramine (Redux) was approved in 1996 by the Food and Drug Administration for use as an appetite suppressant in the management of obesity in the United States. In the 5 months after its release, 1.2 million prescriptions were filled (Fishman, 1999). However, it was discovered that long-term use of anorexigen agent (+)-fenfluramine to control body weight resulted in primary pulmonary hypertension (Abenaim et al., 1996), valvular heart disease (Conolly et al., 1997), and systemic hypertension in some populations (Mahadeje, 1974). Studies have shown that the metabolite of (+)-fenfluramine, (+)-norfenfluramine, is an important and involved compound in (+)-fenfluramine-induced cardiovascular system toxicity (Fitzgerald et al., 2000; Rothman et al., 2000; Hong et al., 2004; Ni et al., 2004a). In this study, we investigated a possible mechanism by which (+)-norfenfluramine induced vasoconstriction, namely, whether (+)-norfenfluramine caused 5-HT release to induce vasoconstriction.

The Presence of 5-HT and Functional 5-HTT in Mouse Aorta. Figure 1A shows that basal levels of 5-HT and 5-HIAA in mouse aorta are detectable. Pargyline-treatment abolished 5-HIAA content and increased 5-HT concentration in normal mouse aorta (Fig. 1A, no significance), suggesting that similar to the rat aorta (Ni et al., 2004b), 5-HT in mouse aorta is in a metabolically active intracellular location.

Western analysis using whole tissue protein and immunoprecipitated protein shows clearly that the 5-HTT protein exists in mouse aorta. Immunohistochemistry experiment results localized the 5-HTT in mouse aorta smooth muscle and endothelial cell layer. Staining in endothelium is consistent with previous findings in rats (Ni et al., 2004b). Uptake assays indicated that aorta from wild-type (paired with Tph1+/− mice) and C57BL6 have the ability to take up 5-HT (Figs. 3 and 6), suggesting a functional 5-HTT in mouse aorta. The uptake of 5-HT in Tph1+/− mouse aorta ensured this mouse model has functional 5-HTT, which is important for testing our hypothesis. To our knowledge, this is the first time a functional 5-HTT in mouse aorta has been described.

Mechanism by Which (+)-Norfenfluramine Acts as a Vasoactive Agent. TPH is the rate-limiting enzyme in biosynthesis of 5-HT. The 5-HT in periphery is primarily synthesized by TPH 1 and in brain by TPH 2. Tph1+/− mice showed normal 5-HT levels in the brain and an almost depleted 5-HT content in periphery (Walther et al., 2003a). We confirmed these findings here because aortic 5-HT and 5-HIAA levels were markedly decreased in aorta from Tph1+/− mice compared with wild-type mice (Fig. 3).

We hypothesized that a portion of (+)-norfenfluramine-induced vasoconstriction was indirect and dependent on endogenous 5-HT release and subsequent activation of 5-HT2A receptors. To test this hypothesis, we used aorta from Tph1+/− mice. (+)-Norfenfluramine caused concentration-dependent contraction in aorta from Tph1+/− mice, and the potency and maximal contraction were similar to that caused in aorta from wild-type mice. Thus, it is unlikely that activation of 5-HT2A receptors by the release of endogenous 5-HT release played a role in (+)-norfenfluramine-induced contraction.

It has been reported that (+)-norfenfluramine has affinity with 5-HT2A receptors (Ki = 1516 nM), and also function studies showed that it is a relatively potent partial agonist at 5-HT2A receptors (Kact = 630 ± 141 nM) (Rothman et al., 2000). The 5-HT2A receptor antagonist ketanserin competitively antagonized the (+)-norfenfluramine contraction in aorta from Tph1+/− mice (Fig. 4B). Although it has been reported that high concentrations of ketanserin have an effect on α1-adrenergic receptor activation, the concentration we used in this study (3 nM) should minimally affect the α1-adrenergic receptor (ketanserin at 5-HT2A receptor, Ki = 0.39 nM; Lysen et al., 1982; and ketanserin at α1-adrenergic receptor, Ki = 72.4 nM; Korstanje et al., 1986). 5-HT2C receptors, another receptor for which ketanserin has significant affinity, have never definitively been found in periphery (Barnes and Sharp, 1999). According to our data, (+)-norfen-
fluoxetine may directly activate 5-HT$_{2A}$ receptor and cause vasoconstriction.

The (+)-norfenfluramine-induced contraction in aorta from Tph1$^{-/-}$ mice was also inhibited by 5-HTT inhibitor fluoxetine (Fig. 4C). Fluoxetine functions by binding to transporter proteins and blocking transporter-mediated recapture of 5-HT. 5-HTT mediates the transport of fenfluramine and (+)-norfenfluramine into the presynaptic nerve terminal for 5-HT releasing (Rothman and Baumann, 2002). In theory, the 5-HT receptor inhibitor fluoxetine should inhibit the effect of 5-HTT substrate-type releasing agent, in our case (+)-norfenfluramine (for review, see Rothman and Baumann, 2002). Thus, there are two possible explanations for our results. First, (+)-norfenfluramine may have intracellular function, which is important for vasoconstriction. A speculation is that after (+)-norfenfluramine was transferred into cytoplasm by 5-HTT, it exerts its functions and changes vasoactivity. Fluoxetine could inhibit (+)-norfenfluramine-induced vasoconstriction by inhibiting the intracellular movement of (+)-norfenfluramine. (+)-Norfenfluramine may have other intracellular functions than releasing 5-HT, and those functions could be important to (+)-norfenfluramine-induced contraction. Recently, Walther et al. (2003b) reported that intracellular 5-HT transamidated small GTPases by transglutaminases during activation and aggregation of platelets, resulting in GTPases being constitutively activate. (+)-Norfenfluramine has similar chemical structure with 5-HT, making intracellular functioning of (+)-norfenfluramine possible. A second possibility is that (+)-norfenfluramine induces vasoconstriction directly by activating 5-HT$_{2A}$ receptor and that fluoxetine inhibits (+)-norfenfluramine-induced contraction by acting as a 5-HT$_{2A}$ receptor antagonist. The latter is a reasonable action to suggest.

To distinguish between these two possible mechanisms discussed above, we next used 5-HTT-targeted mutation mice. 5-HTT targeted mutation mouse has a disrupted 5-HTT gene (sl6a4) on C57BL/6 background. 5-HTT function was abolished, as Bengel et al. (1998) observed a deficiency of high-affinity [$^3$H]5-HT uptake in 5-HTT-targeted mutation mice (Bengel et al., 1998). Consistently, the behavioral effects of fluoxetine were lost in 5-HTT-targeted mutation mice (Holmes et al., 2002). Aorta from these animals provided the ideal model to test our hypothesis.

(+)-Norfenfluramine induced a concentration-dependent contraction in aorta from 5-HTT-targeted mutation mice (Fig. 5A), indicating the independence of (+)-norfenfluramine from 5-HTT. Fluoxetine inhibited (+)-norfenfluramine-induced contraction in aorta from 5-HTT-targeted mutation mouse, suggesting that fluoxetine has other effects than inhibiting 5-HTT in mouse aorta. Contraction to 5-HT in the mouse aorta is mediated primarily by a 5-HT$_{2A}$ receptor (McKune and Watts, 2001). Literature searches revealed that the affinity of fluoxetine at rat and human 5-HT$_{2A}$ receptor ranges from $K_i = 141$ to 708 nM, but there is no report of fluoxetine affinity at the mouse 5-HT$_{2A}$ receptor. Our observation of the inhibitory effect by fluoxetine on 5-HTT-induced contraction on aorta from 5-HTT-targeted mutation mouse suggests that fluoxetine inhibits the 5-HT$_{2A}$ receptor. An ideal experiment would be to test the effect of fluoxetine on a 5-HT$_{2A}$ receptor-specific agonist that could not act as a substrate for 5-HTT, but such an agonist has proved difficult to find.

Since we observed a different effect of fluoxetine and fluvoxamine on (+)-norfenfluramine-induced (Fig. 2) and also 5-HTT-induced mouse aortic contraction (data not shown), we questioned whether fluoxetine and fluvoxamine had different effects on 5-HTT inhibition. Fluvoxamine but not fluoxetine exerted inhibition on 5-HT uptake in mouse aorta (Fig. 6). This is different from what we observed in rat superior mesenteric arteries, in which both fluoxetine (Ni et al., 2004b) and fluvoxamine (data not shown) inhibited 5-HT uptake. Chang et al. (1996) reported that at the nucleotide level, mouse and rat 5-HTT share more than 90% overall homology (94% in the coding region, 74% in the 3’ untranslated region). The difference in the coding regions may important for 5-HTT interacting with 5-HTT inhibitors and may explain the different pharmacological characteristics. In summary, fluoxetine is not a good 5-HTT inhibitor in mouse peripheral arteries because it did not inhibit 5-HT uptake but seems to inhibit 5-HT$_{2A}$ receptor.

Figure 6B shows that, only about 50% of the 5-HT uptake was inhibited by fluvoxamine (1 μM). It is possible that other transporters such as norepinephrine transporter (NET), dopamine transporter, organic cation transporter (OCT) 1, and/or OCT 3 also involved in this uptake. In the rat, we have demonstrated that the NET inhibitor nisoxetine did not reduce 5-HT uptake in superior mesenteric arteries (Ni et al., 2004b). Thus, we do not think NET mediates 5-HT uptake in peripheral arteries. Presently, there is no evidence suggesting the presence of dopamine transporter, OCT 1, and OCT 3 in peripheral arteries. Rothman et al. (2003) reported that (+)-norfenfluramine is a potent substrate for NET and releases NE (Rothman et al., 2003). It is feasible to hypothesize that (+)-norfenfluramine-induced arterial contraction is dependent on release of NE and activation of α$_1$-adrenergic receptors. However, it is unlikely the sympathetic nervous system played a role in (+)-norfenfluramine-induced contraction as we observed and reported previously that 1) a similar contraction in arteries from normal and 6-hydroxydopamine-denervated rats; and 2) the α$_1$-adrenergic receptor antagonist prazosin and α$_2$-adrenergic receptors antagonist RX821002 (2-methoxydazoxan) were unable to block (+)-norfenfluramine-induced contraction in aorta (Ni et al., 2004a).

Knowledge of 5-HTT in Peripheral Artery. The intention of this study was to determine the possible mechanism by which (+)-norfenfluramine causes vasoconstriction. The newly found 5-HTT in peripheral arteries may or may not be the same 5-HTT in brain in terms of amino acid sequence, structure, and pharmacological characteristics. It is well established that (+)-fenfluramine and (+)-norfenfluramine are 5-HT releasers in brain (for review, see Rothman and Baumann, 2002). It was reported recently that (+)-norfenfluramine released 5-HT from rat ileum (Rezaie-Majd et al., 2004), but it also has been reported by the same group that (+)-norfenfluramine inhibits 5-HT uptake and has no effect on 5-HT efflux in intact human platelet (Johnson et al., 2003). Thus, 5-HTT may function differently in different tissues, and significant species-based differences in 5-HTT function may exist. Our collective data in rat and mouse suggest a real difference in how fluoxetine interacts with the arterial 5-HTT. The investigation of the effects of (+)-norfenfluramine on arterial smooth muscle will also help us understand the function of 5-HTT in the periphery. If the 5-HTT in artery...
is different from the classic neuronal 5-HTT, the peripheral 5-HTT might become a specific drug target.

Summary. We observed that (+)-norfenfluramine-induced contraction was not dependent on endogenous 5-HT or 5-HTT in peripheral arteries but that it may directly activate 5-HT₂, receptor. Moreover, fluoxetine as a 5-HTT inhibitor may not be a clean drug for study of 5-HTT functions in peripheral mouse arteries.

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References


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