JPET Fast Forward. Published on May 18, 2005 as DOI: 10.1124/jpet.105.087080

JPET #87080

(+)-Norfenfluramine-induced arterial contraction is not dependent on endogenous 5-HT or 5-HTT

Wei Ni, Claudia S. Wilhelm, Michael Bader, Dennis L. Murphy, Keith Lookingland and Stephanie W. Watts

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824-1317, USA. (WN, KL, SWW)

Max Delbrück Center for Molecular Medicine (MDC), Robert-Rossle-Strasse 10, D-13092, Berlin-Buch, Germany. (CSW, MD)

Murphy DL: Laboratory of Clinical Science, Building 10, Room 3D41, 10 Center Drive, MSC 1264, NIMH, NIH, Bethesda, MD 20892, USA. (DLM)

JPET #87080

Running title: (+)-Norfen vasoconstriction independent of serotonin

Corresponding Author:

Wei Ni, BS

B445 Life Sciences Building

Department of Pharmacology & Toxicology

Michigan State University

East Lansing, MI 48824-1317

Telephone: (517) 353-3900

Telefax: (517) 353-8195

E-mail: niwei@msu.edu

Number of text pages: 27

Number of tables: 0

Number of figures: 6

Number of references: 27

Number of words in the Abstract: 243

Number of words in the Introduction: 347

Number of words in the Discussion: 1679

Abbreviation list:

5-hydroxyindole acetic acid	5-HIAA
5-Hydroxytryptamine	5-HT
5-Hydroxytryptamine transporter	5-HTT
Dopamine transporter	DAT
Norepinephrine transporter	NET
Organic cation transporter	OCT
Phenylmethylsulfonyl fluoride	PMSF
Phosphate buffered saline	PBS
Physiological salt solution	PSS
Primary pulmonary hypertension	PPH
Standard error of the mean	SEM
Sympathetic nervous system	SNS
Tris buffered saline	TBS
Tryptophan hydroxylase	TPH

Recommended section: Cardiovascular

Abstract

(+)-Norfenfluramine, the major metabolite of fenfluramine, causes vasoconstriction dependent on the 5-HT_{2A} receptor in rat. (+)-Norfenfluramine was reported as a 5-hydroxytryptamine transporter (5-HTT) substrate and 5-hydroxytryptamine (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-HT_{2A} 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). 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 EC_{50} [M], wild type=5.73±0.02, *Tph1-/-*=5.62±0.09), and these contractions were inhibited by the 5-HT_{2A} receptor antagonist ketanserin (3 nM) by a similar magnitude in aorta from wild type and *Tph1-/-* (wild type=19.4, *Tph1-/-*=15.4-fold rightward shift *vs.* control), as did fluoxetine (1 μM) (wild type=22.4, *Tph1-/-*=28.8-fold rightward shift *vs.* 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 contraction 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-HT_{2A} receptors directly. The 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.

Introduction

The highly effective anorexigen (+)-fenfluramine (Redux®) was widely prescribed for the treatment of obesity until it was associated with primary pulmonary hypertension (PPH) (Abenhaim et al.,1996) and aortic valvular disease (Connolly et al.,1997). We have reported previously that the hepatic de-ethylated metabolite of (+)-fenfluramine, (+)-norfenfluramine, is vasoactive. (+)-Norfenfluramine causes contraction 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., 2004 a). Recently, we found that 5-HT and a functional 5-HTT are present in rat peripheral arteries (Ni et al., 2004 b). 5-HT is a vasoconstrictor and causes contraction in many arteries and veins, especially conduit vessels *via* 5-HT_{2A} receptors (Martin, 1994). (+)-Fenfluramine and (+)-norfenfluramine are 5-HTT substrates and potent 5-HT releasers (Garattini et al., 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 et al., 1999), we hypothesized that (+)-norfenfluramine-induced vasoconstriction is dependent on release of endogenous 5-HT *via* 5-HTT with consequent 5-HT-induced activation of 5-HT_{2A} receptor mediating 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. Lastly, we studied the different effects of fluoxetine and fluvoxamine on 5-HT uptake in mouse aorta.

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, MI), *Tph1-/-* mice and wild type male mice (C57BL/6; 20-24 g; Max Delbrück Center for Molecular Medicine, Germany), 5-HTT targeted mutation mice and wild type male mice (C57BL/6; 30-40 g; National Institute of Mental Health, MD) were used in the experiments.

HPLC isolation of 5-HT, 5-HIAA:

Aorta from vehicle or pargyline (i.p., 100 mg/kg, 30 min)–treated mice were dissected, cleaned and placed in tissue buffer [0.05 mM sodium phosphate & 0.03 mM citric acid buffer (pH 2.5) containing 15% methanol]. Samples were frozen in –80 ^{o}C until assay. Samples were thawed, sonicated for 3 seconds and centrifuged for 30 seconds (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-hydroxyindole acetic acid (5-HIAA) and 5-HT in tissue supernatants were determined by isocratic high pressure liquid chromatography (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, 10% glycerol] with protease inhibitors (0.5 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin). Homogenates were centrifuged (11,000 g for 10 min, 4ºC) and supernatant total protein measured.

Immunoprecipitation: Mouse aorta protein homogenates (200 µg) were incubated with 2 µg/ml 5-HTT C-20 antibody (Santa Cruz, CA, USA) for 2 hour. Twenty microliters of protein A/G-Agarose were added and tumbled overnight at 4ºC. Beads were collected by centrifugation at 2,500 rpm for 30 sec and supernatant discarded. Beads were washed 3 times with phosphate buffered saline (PBS), each time repeating the centrifugation step above. After the final wash, the beads were boiled in 40 µL of 2X lysis buffer, centrifuged and supernatant loaded on SDS polyacrylamide gel for Western analysis.

Western blotting: Fifty micrograms of total protein or 10 µL of immunoprecipitated samples were separated on 10% SDS polyacrylamide gels using a Mini Bio-Rad III apparatus. Membranes were blocked for three hours in 4% chick egg ovalbulmin [4 ^{o}C, Tris buffered saline (TBS)-0.1% Tween + 0.025% NaN_3]. Primary antibody (0.5 µg/ml, 5-HTT C-20, Santa Cruz, CA, USA) was incubated with blots overnight at 4 ^{o}C. Blots were then rinsed thrice in TBS + Tween (0.1%) with a final rinse in TBS and incubated with donkey peroxidase-linked anti-goat secondary antibody (1:2,000, Santa Cruz, CA, USA) for 1 hour at 4 ºC with

rocking. ECL® reagents (Amersham Life Sciences, Arlington Heights, IL, USA) were used to visualize bands.

Immunohistochemistry:

Mouse aorta was snap frozen in OCT compound and stored at –70^{o}C until use. Arterial sections (8 micron) were cut and air dried (overnight, room temp). Samples were cold acetone fixed, washed 3 times with PBS, and endogenous peroxidase blocked [0.3% H_2O_2 in PBS for 30 minutes]. Sections were blocked for non-specific binding in PBS containing 1.5% of competing serum. In a humidified chamber, samples incubated 24 hours with antibody (5-HTT C-20, Santa Cruz, CA; 4 ^{o}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, USA). Sections were washed 3 times with PBS and incubated with a peroxidase-conjugated secondary antibody (30 minutes, room temp). Samples were washed and incubated with Vectastain® ABC Elite reagent (30 minutes, room temp) followed by 3,3'-diaminobenzidine (DAB)/H_2O_2. 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 PSS. Vessels were trimmed of fat, cut into helical strips (0.15X0.75cm). Tissues were attached to a fixed, stainless steel rod at one end and to a force transducer at the other. Baths were filled with physiological salt solution (PSS, 103 mM NaCl; 4.7 mM KCL; 1.18 mM KH_2PO_4; 1.17 mM $MgSO_4$-$7H_2O$; 1.6 mM $CaCl_2$-$2H_2O$; 14.9 mM $NaHCO_3$; 5.5 mM Dextrose, and 0.03 mM $CaNa_2$ EDTA), warmed to 37ºC 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 one hour with frequent buffer changes. Tissues were then challenged with a maximal concentration of the α_1 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^{-8}-10^{-7} M) and once the contraction plateaued, the muscarinic agonist acetylcholine (10^{-6} M) was administered. We observed 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 followed.

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 three minutes. When contraction reached a maximum, the next higher concentration of agonist was added. Contraction to agonist was normalized to the initial maximal contraction to phenylephrine.

Protocol 2: Testing of effect of antagonist or inhibitor on (+)-norfenfluramine-induced contraction

Vehicle or antagonist/inhibitor was added to the bath for 1 hour. 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 minutes. Tissues were then briefly dipped in drug-free PSS and placed in 75 µL of tissue buffer. Samples were frozen (-80 ^{o}C) until assay. Tissues were incubated with vehicle or 5-HTT inhibitor 30 minutes prior to 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 % of vehicle group in 5-HT uptake experiments. Band density quantitation in Western analysis was performed using NIH Image (v.1.61). When comparing 2 groups, unpaired Student t-tests were used as control and treated samples were different tissues. Contractile data are expressed as ± standard error of the mean (SEM) and 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 EC_{50} values were calculated using a nonlinear regression analysis using the algorithm [effect = maximum response /1+(EC_{50}/agonist concentration)] in the program GraphPad® Prism. Apparent antagonist dissociation constants (K_B values) were calculating using the following equation (1):

(1) $$\log (dr-1) = \log [B] - \log K_B$$

where dr is the EC_{50} value of agonist in the presence of the antagonist divided by the EC_{50} value of agonist in the absence of the antagonist, [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 Chemical Co. (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 [^{3}H] 5-HT, which only can measure 5-HT but not its metabolite. We used HPLC-based measurements of arterial 5-HT and 5-HIAA on an HPLC system that allowed us concurrent measurement of amines in the same samples. We measured basal level of 5-HT (1.39 $\pm$ 0.24 ng/mg protein) and the monoamine oxidase (MAO) -A metabolite 5-HIAA (0.27 $\pm$ 0.04 ng/mg protein) in normal C57BL6 mouse aorta (Figure 1 A). 5-HIAA production was abolished and 5-HT concentration increased (no significance) in aorta from pargyline-treated mice (5-HT= 2.03 $\pm$ 0.38 ng/mg protein; 5-HIAA= 0.015 $\pm$ 0.009 ng/mg protein, Figure 1 A). 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 recognizing 5-HTT C terminus (C-20, Santa Cruz, CA, USA) was performed in mouse aorta whole tissue homogenate supernatant (Figure 1 B, homog) and immunoprecipitated protein (Figure 1 B, IP). These bands in both straight homogenate or immunoprecipitated protein samples migrated at ~ 70 kDa in mass, consistent with that reported for brain and lung 5-HTT (between 60-80 kDa).

Immunohistochemical experiments using the same antibody localized the 5-HTT protein to smooth muscle and the endothelial layer of the mouse aorta

JPET Fast Forward. Published on May 18, 2005 as DOI: 10.1124/jpet.105.087080
This article has not been copyedited and formatted. The final version may differ from this version.

compare left and middle panel of figure 1 C. The black staining in adventitia was not competed off by competing peptide, thus this staining is likely non-specific. 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 responsive curve to (+)-norfenfluramine in aorta from C57BL/6 mice. Similar as observed in rat aorta (Ni et al., 2004 a), (+)-norfenfluramine contracted the mouse aorta in a concentration-dependent manner with a $-\log EC_{50}$ 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 ($-\log EC_{50}$ value [M] = 4.39 ± 0.04, pK_B Value = 7.29±0.06) by fluoxetine (1 µM). However, a different 5-HTT inhibitor fluvoxamine showed no effect on (+)-norfenfluramine-induced aortic contraction ($-\log EC_{50}$ 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.

JPET Fast Forward. Published on May 18, 2005 as DOI: 10.1124/jpet.105.087080
This article has not been copyedited and formatted. The final version may differ from this version.

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., 2003 a). Figure 3 shows 5-HIAA and 5-HT concentrations in aorta from *Tph1-/-* mice with vehicle or with exogenous 5-HT (1 μM, 15 minutes). 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 to 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 examined 5-HT-uptake. Aorta from wild type and *Tph1-/-* mice showed similar ability to uptake exogenous 5-HT. After incubation with exogenous 5-HT (1 μM) for 15 minutes, 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, Figure 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-/-* mouse 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 EC_{50} value [M] = 6.90 ± 0.03) that was similar with that in wild type mice (–log EC_{50} value [M] = 6.96 ± 0.03) (Figure 4 A). Thus, aorta 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 EC_{50} value [M] = 5.62±0.09 (Figure 4 B C). The potency of (+)-norfenfluramine-induced contractions was similar in aorta from wild type and *Tph1-/-* mice –log EC_{50} value [M] = 5.74±0.02). These data suggest that (+)-norfenfluramine-induced contraction is independent of endogenous 5-HT release.

Effect of 5-$HT_{2A/2C}$ receptor antagonist ketanserin and 5-HTT inhibitor fluoxetine on (+)-norfenfluramine-induced contraction in aorta from *Tph1-/-* mice

To determine the role of 5-HT_{2A} receptor and 5-HTT in (+)-norfenfluramine-induced contraction, mouse aorta were examined in the presence of 5-HT_{2A} 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 (Figure 4 B). The apparent dissociation constant calculated from this shift (pK_B = 9.79± 0.13) is consistent with antagonism of the 5-HT_{2A} 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_B value= 9.67 ± 0.17 (Figure 4 B), similar to the pK_B 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 ± 0.07, pK_B value= 7.27±0.19, Figure 4 C) in aorta from *Tph1-/-* mice aorta, similar to what we observed in wild type mice paired with *Tph1-/-* mice and C57BL/6 mice from Charles River, MI (Figure 2). Thus, the 5-HT_{2A} receptor and potentially the 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 determine whether effects of fluoxetine are 5-HTT-dependent. In aorta isolated from wild type and 5-HTT KO, cumulative (+)-norfenfluramine concentration responsive curve were generated in the presence of vehicle or fluoxetine (1 μM). (+)-Norfenfluramine induced a concentration dependent contraction in both wild type and 5-HTT KO (-log EC_{50} [M], wild type= 5.54±0.03; 5-HTT KO= 5.52±0.14, Figure 5 A). 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 5 B shows 5-HT-induced concentration dependent mouse aortic contraction (-log EC_{50} [M], wild type= 6.93±0.11; 5-HTT KO= 6.99±0.12) was inhibited by fluoxetine (1 μM) in wild type and 5-HTT KO (-log EC_{50} [M], wild type= 5.63±0.10; 5-HTT KO= 5.46±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 MAO-A and thus inhibited 5-HT metabolite to 5-HIAA. Aortic 5-HIAA concentrations were almost abolished after treated with pargyline and thus we reported and only compared 5-HT concentrations. Fluoxetine (1 μM) alone moderately increased 5-HT concentrations (not significantly different from control) and did not inhibit 5-HT uptake in mouse aorta (Figure 6 A). By contrast, fluvoxamine (1 μM) had no effect on basal level 5-HT and significantly reduced 5-HT uptake in mouse aorta (Figure 6 B). Thus, fluoxetine and fluvoxamine had different effects on 5-HT uptake in mouse aorta.

Discussion

(+)-Fenfluramine (Redux) was approved in 1996 by FDA for use as an appetite suppressant in the management of obesity in USA. 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 PPH (Abenhaim et al., 1996), valvular heart disease (Connolly et al., 1997) and systemic hypertension in some populations (Mabadeje, 1974). Studies have shown that the metabolite of (+)-fenfluramine, (+)-norfenfluramine, is an important and involved compound in (+)-fenfluramine-induced cardiovascular system toxicity (Rothman et al., 2000; Fitzgerald et al., 2000; Hong et al., 2004; Ni et al., 20004 a). 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 1 A 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 (Figure 1 A, no significance), suggesting that similar to the rat aorta (Ni et al., 2004 b), 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., 2004 b). Uptake assays indicated that aorta from wild type (paired with *Tph1-/-* mice) and C57BL6 have the ability to uptake 5-HT (Figure 3, 6), suggesting a functional 5-HTT in mouse aorta. The uptake of 5-HT in *Tph1-/-* mouse aorta ensured this mice 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., 2003 a). We confirmed these findings presently as aortic 5-HT and 5-HIAA levels were markedly decreased in aorta from *Tph1-/-* mice compared to wild type mice (Figure 3).

We hypothesized that a portion of (+)-norfenfluramine-induced vasoconstriction was indirect, dependent on endogenous 5-HT release and subsequent activation of 5-HT_{2A} 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-HT_{2A} 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-HT_{2A} receptors (K_i=1516 nM) and also function studies showed that it is a relatively potent partial agonist at 5-HT_{2A} receptors (Kact = 630±141 nM) (Rothman *et al.*, 2000). The 5-$HT_{2A/2C}$ receptor antagonist ketanserin competitively antagonized the (+)-norfenfluramine contraction in aorta from *Tph1-/-* mice (Figure 4 B). Although it has been reported that high concentrations of ketanserin has effect on α_1 adrenergic receptor activation, the concentration we used in this study (3 nM) should minimally affect the α_1 adrenergic receptor (ketanserin at 5-HT_{2A} receptor, Ki = 0.39 nM, Leysen *et al.,* 1982; ketanserin at α_1 adrenergic receptor, Ki = 72.4 nM, Korstanje *et al.,* 1986). 5-HT_{2C} receptors, another receptor for which ketanserin has significant affinity, have never definitively been found in periphery (Barnes and Sharp, 1999). According to our data, (+)-norfenfluramine 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 (Figure 4 C). 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 reuptake inhibitor fluoxetine should inhibit the effect of 5-HTT substrate-type releasing agent, in our case (+)-norfenfluramine (for review, 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.* reported that intracellular 5-HT transamidated small GTPases by transglutaminases during activation and aggregation of platelets, resulting in GTPases being constitutively activate (Walther et al., 2003 b). (+)-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 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 (slc6a4) on C57BL/6 background. 5-HTT function was abolished as Bengel *et al*. 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 mouse (Figure 5 A) indicating the independence of (+)-norfenfluramine from 5-HTT. Fluoxetine inhibited (+)-norfenfluramine-induced contraction in aorta from 5-HTT targeted mutation mouse aorta, 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 range from Ki = 141 nM 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-HT-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 (Figure 2) and also in 5-HT-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 (Figure 6). This is different from what we observed in rat superior mesenteric arteries, in which both fluoxetine (Ni et al., 2004 b) and fluvoxamine (data not shown) inhibited 5-HT uptake. Chang *et al*.

JPET Fast Forward. Published on May 18, 2005 as DOI: 10.1124/jpet.105.087080
This article has not been copyedited and formatted. The final version may differ from this version.

reported that at the nucleotide level, mouse and rat 5-HTT share over 90% of overall homology (94% in the coding region; 74% in the 3' untranslated region; Chang et al., 1996). 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 appears to inhibit 5-HT_{2A} receptor.

Figure 6 B shows that, only about 50% of the 5-HT uptake were inhibited by fluvoxamine (1 μM). It is possible that other transporters such as norepinephrine transporter (NET), dopamine transporter (DAT), 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., 2004 b). Thus, we do not believe NET mediates 5-HT uptake in peripheral arteries. Presently, there is no evidence suggesting the presence of DAT, OCT 1 and OCT 3 in peripheral arteries.

Rothman *et al.* reported that (+)-norfenfluramine is a potent substrate for NET and releases NE (Rothman et al., 2003). It is feasible to hypothesis that (+)-norfenfluramine-induced arterial contraction 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 before that (1) a similar contraction in arteries from normal and 6-OHDA-denervated rats; (2) the α_1-adrenergic receptor antagonist prazosin and α_2-adrenergic receptors antagonist RX821002 (2-methoxyidazoxan)

were unable to block (+)-norfenfluramine-induced contraction in aorta (Ni et al., 2004 a).

Knowledge of 5-HTT in periphery 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, Rothman RB and Baumann MH, 2002). It was reported recently that (+)-norfenfluramine released 5-HT from rat ileum (Rezaie-Majd et al., 2004) but it has also been reported by the same group that (+)-norfenfluramine inhibit 5-HT uptake and has no effect on 5-HT efflux in intact human platelet (Johnson et al., 2003). Thus the 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 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 may directly activate 5-HT_{2A} receptor. Moreover, fluoxetine as a 5-HTT inhibitor may not a clean drug for study of 5-HTT functions in peripheral mouse arteries.

Acknowledgements

We are grateful to the pharmaceutical companies Eli Lilly and SRI International for donating the drugs.

Reference:

Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X, Higenbottam T, Oakley C, Wouters E, Aubier M, Simonneau G and Begaud B (1996) Appetite-suppressant drugs and the risk of primary pulmonary hypertension. *N Engl J Med* **355**: 609-616.

Barnes NM and Sharp T (1999) A review of central 5-HT receptors and their function. *Neuropharmacology* **38**(8):1083-1152.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mossner R, Westphal H, Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. *Mol Pharmacol.* **53**(4):649-55.

Chang AS, Chang SM, Starnes DM, Schroeter S, Bauman AL, Blakely RD (1996) Cloning and expression of the mouse serotonin transporter. *Brain Res Mol Brain Res.* **43**(1-2):185-192.

Chapin DS, Lookingland KJ, Moore KE (1986) Effects of LC mobile phase composition on retention times for biogenic amines, and their precursors and metabolites. *Cur. Separations*. **7**: 68-70.

Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD and Schaff HV (1997) Valvular heart disease associated with fenfluramine-phentermine. *N Engl J Med* **337**: 581-588.

Fishman AP. Aminorex to fen/phen: an epidemic foretold. (1999) *Circulation.* **99**(1):156-161.

Fitzgerald LW, Burn TC, Brown BS, Patterson JP, Corjay MH, Valentine PA, Sun JH, Link JR, Abbaszade I, Hollis JM, Largent BL, Hartig PR, Hollis GF, Meunier PC, Robichaud AJ, Robertson DW (2000) Possible role of valvular serotonin 5-HT 2B receptors in the cardiopathy associated with fenfluramine. *Mol Pharmacol* **57**: 75–81

Flechtner-Mors M, Ditschuneit HH, Yip I and Adler G (1988) Blood pressure and plasma norepinephrine responses to dexfenfluramine in obese postmenopausal women. *Am J Clin Nutr* **67**(4): 611-615.

Garattini S (1995) Biological actions of drugs affecting serotonin and eating. *Obes Res* 3 **suppl 4:** 463S-470S. Review.

Holmes A, Yang RJ, Murphy DL, Crawley JN (2002) Evaluation of antidepressant-related behavioral responses in mice lacking the serotonin transporter. *Neuropsychopharmacology.* **27**(6):914-923.

Hong Z, Olschewski A, Reeve HL, Nelson DP, Hong F, Weir EK (2004) Nordexfenfluramine causes more severe pulmonary vasoconstriction than dexfenfluramine. *Am J Physiol Lung Cell Mol Physiol.* **286**(3):L531-538.

Johnson GJ, Leis LA, Dunlop PC, Weir EK (2003) The effect of the anorectic agent, d-fenfluramine, and its primary metabolite, d-norfenfluramine, on intact human platelet serotonin uptake and efflux. *J Thromb Haemost.* **1**(12):2663-2668.

Korstanje C, Sprenkels R, Doods HN, Hugtenburg JG, Boddeke E, Batink HD, Thoolen MJ, and Van Zwieten PA (1986) Characterization of flufylline, fluprofylline, ritanserin, butanserin and R 56413 with respect to in-vivo alpha 1-,alpha 2-and 5-HT 2-receptor antagonism and in-vitro affinity for alpha 1-,alpha 2- and 5-HT 2-receptors: comparison with ketanserin. *J Pharm Pharmacol* **38**: 374-379

Leysen JE, Niemegeers CJ, Van Nueten JM, and Laduron PM (1982) [3H]Ketanserin (R 41 468), a selective 3H-ligand for serotonin 2receptor binding sites. Binding properties, brain distribution and functional role. *Mol Pharmacol* **21**: 301-314.

Mabadeje AF (1974) Proceedings: Fenfluramine-induced hypertension. *West Afr J Pharmacol Drug Res* **2**(1):54.

Martin GR (1994) Vascular receptors for 5-hydroxytryptamine: distribution, function and classification. *Pharmacol Ther* **62**(3): 283-324.

McKune CM, Watts SW (2001) Characterization of the serotonin receptor mediating contraction in the mouse thoracic aorta and signal pathway coupling. *J Pharmacol Exp Ther.* **297**(1):88-95.

Ni W, Li MW, Thakali K, Fink GD, Watts SW (2004a) The fenfluramine metabolite (+)-norfenfluramine is vasoactive. *J Pharmacol Exp Ther.* **309**(2):845-852.

Ni W, Thompson JM, Northcott CA, Lookingland K, Watts SW (2004b) The serotonin transporter is present and functional in peripheral arterial smooth muscle. *J Cardiovasc Pharmacol.* **43**(6):770-781.

Rezaie-Majd S, Murar J, Nelson DP, Kelly RF, Hong Z, Lang IM, Varghese A, Weir EK (2004) Increased release of serotonin from rat ileum due to dexfenfluramine. *Am J Physiol Regul Integr Comp Physiol.* **287**(5):R1209-1213.

Rothman RB, Baumann MH, Savage JE, Rauser L, McBride A, Hufeisen SJ and Roth BL (2000) Evidence for possible involvement of 5-HT(2B) receptors in the cardiac valvulopathy associated with fenfluramine and other serotonergic medications. *Circulation* **102**(23): 2836-2841.

Rothman RB, Baumann MH (2002) Therapeutic and adverse actions of serotonin transporter substrates. *Pharmacol Ther.* **95**(1):73-88. Review.

Rothman RB, Vu N, Partilla JS, Roth BL, Hufeisen SJ, Compton-Toth BA, Birkes J, Young R, and Glennon RA (2003) (+)-Fenfluramine and its major metabolite, (+)-norfenfluramine, are potent substrates for norepinephrine transporters. *J Pharmacol Exp Ther* **305**: 1191-1199

Russell A and Watts SW (2000) Vascular Reactivity of Isolated Thoracic Aorta of the C57BL/6J Mouse. *J Pharmacol Exp Ther* **294**(2):598-604.

Walther DJ, Peter JU, Bashammakh S, Hortnagl H, Voits M, Fink H, Bader M (2003a) Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science.* **299**(5603):76.

Walther DJ, Peter JU, Winter S, Holtje M, Paulmann N, Grohmann M, Vowinckel J, Alamo-Bethencourt V, Wilhelm CS, Ahnert-Hilger G, Bader M (2003b) Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release. *Cell.* **115**(7):851-862.

Footnotes:

Source of financial support: NIH (HL58489), AHA 0415397Z

Corrspondence address:

Wei Ni, BS

B445 Life Sciences Building

Department of Pharmacology & Toxicology

Michigan State University

East Lansing, MI 48824-1317

Telephone: (517) 353-3900

Telefax: (517) 353-8195

E-mail: niwei@msu.edu

Figure Legends

Figure 1. A: Concentration of 5-HT and 5-HIAA in aorta from mice treated with vehicle or pargyline. Bars represent means and vertical bars the SEM for the number of animals indicated in parentheses. * indicates statistical difference ($p<0.05$) *vs* saline- treated mice within each group. B: Western blot of 5-HTT in tissue homogenates supernatant and immunoprecipitated protein (by 5-HTT antibody C-20) from aorta from normal C57BL/6 mice. Representative of four different mice. C. Immunohistochemical staining of the 5-HTT (C-20 antibody) in mouse aorta smooth muscle between cables of elastin/collage. Parallel sections were incubated with antibody alone (left), quenched with a 5X excess competing peptide (middle) or no primary (right). Red arrows indicate the location of staining. Representative of four different experiments. 5-HT= 5-Hydroxytryptamine, 5-HIAA= 5-Hydroxyindole acetic acid, 5-HTT= 5-Hydroxytryptamine transporter, Homog.= homogenates, IP= immunoprecipitated protein, CP = competing peptide (5X excess).

Figure 2. (+)-Norfenfluramine concentration response curve and effects of fluoxetine (1 µM) and fluvoxamine (1 µM) on (+)-norfenfluramine-induced contraction in aorta from normal C57BL6 mice. Points represent means and vertical bars the SEM for the number of animals indicated in parentheses. * indicate statistical differences ($p<0.05$) *vs* vehicle. PE = phenylephrine.

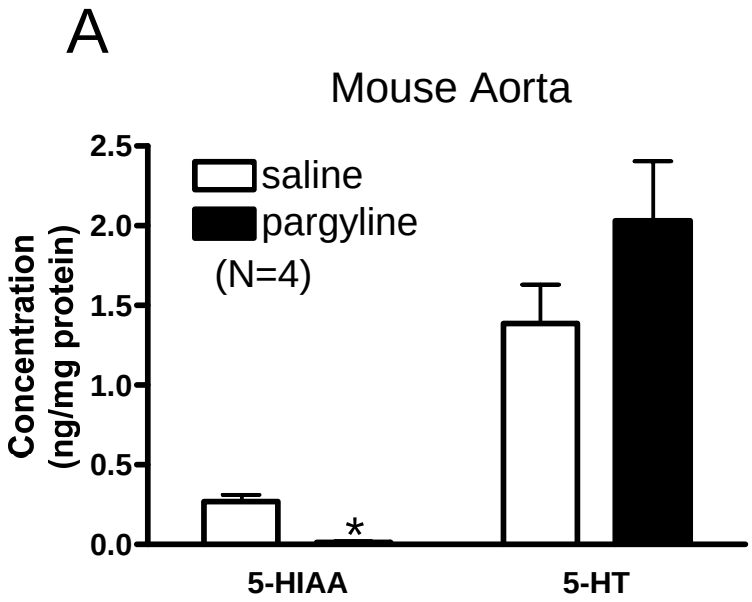
Figure 3. 5-HT and 5-HIAA concentration in wild type and *Tph1-/-* mouse aorta incubated with vehicle or 5-HT (1 µM) for 15 minutes. Bars represent means and vertical bars the SEM for the number of animals indicated in parentheses. * indicate statistical differences ($p<0.05$) *vs* wild type within each group (5-HT, 5-HIAA). # indicate statistical differences ($p<0.05$) *vs* basal level within each group (5-HT, 5-HIAA). 5-HT= 5-hydroxytryptamine. 5-HIAA= 5-hydroxyindole acetic acid.

Figure 4. A: 5-HT concentration response curve in aorta from wild type and *Tph1-/-* mice. B: Effect of 5-HT_{2A} receptor antagonist ketanserin (3 nM) on (+)-norfenfluramine-induced contraction in aorta from wild type mice and *Tph1-/-* mice. C: Effect of 5-HTT inhibitor fluoxetine (1 µM) on (+)-norfenfluramine-induced contraction in aorta from wild type mice and *Tph1-/-* mice. Points represent means and vertical bars the SEM for the number of animals indicated in parentheses. * indicate statistical differences ($p<0.05$) *vs* control in wild type mice. # indicate statistical differences ($p<0.05$) *vs* control in *Tph1-/-* mice. PE = phenylephrine. 5-HT= 5-hydroxytryptamine.

Figure 5. A: Effect of 5-HTT inhibitor fluoxetine (1 µM) on (+)-norfenfluramine-induced cumulative contraction in aorta from wild type mice and 5-HTT targeted mutation mice. B: Effect of fluoxetine (1 µM) on 5-HT-induced contraction in aorta from wild type mouse and 5-HTT targeted mutation mouse. Points represent means and vertical bars the SEM for the number of animals indicated in

parentheses. * indicate statistical differences ($p<0.05$) *vs* vehicle in wild type mice. # indicate statistical differences ($p<0.05$) *vs* vehicle in 5-HTT targeted mutation mice. PE = phenylephrine. 5-HT= 5-hydroxytryptamine.

Figure 6. A: Effect of 5-HTT inhibitor fluoxetine (1 μM) on 5-HT-uptake in aorta from pargyline-treated mice. B: Effect of 5-HTT inhibitor fluvoxamine (1 μM) on 5-HT-uptake in aorta from pargyline-treated mice. Bars represent means and vertical bars the SEM for the number of animals indicated in parentheses. * indicate statistical differences ($p<0.05$) *vs* vehicle. # indicate statistical differences ($p<0.05$) *vs* 5-HT (1 μM). 5-HT= 5-hydroxytryptamine.



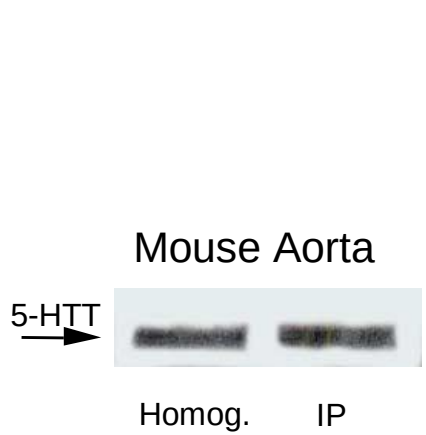
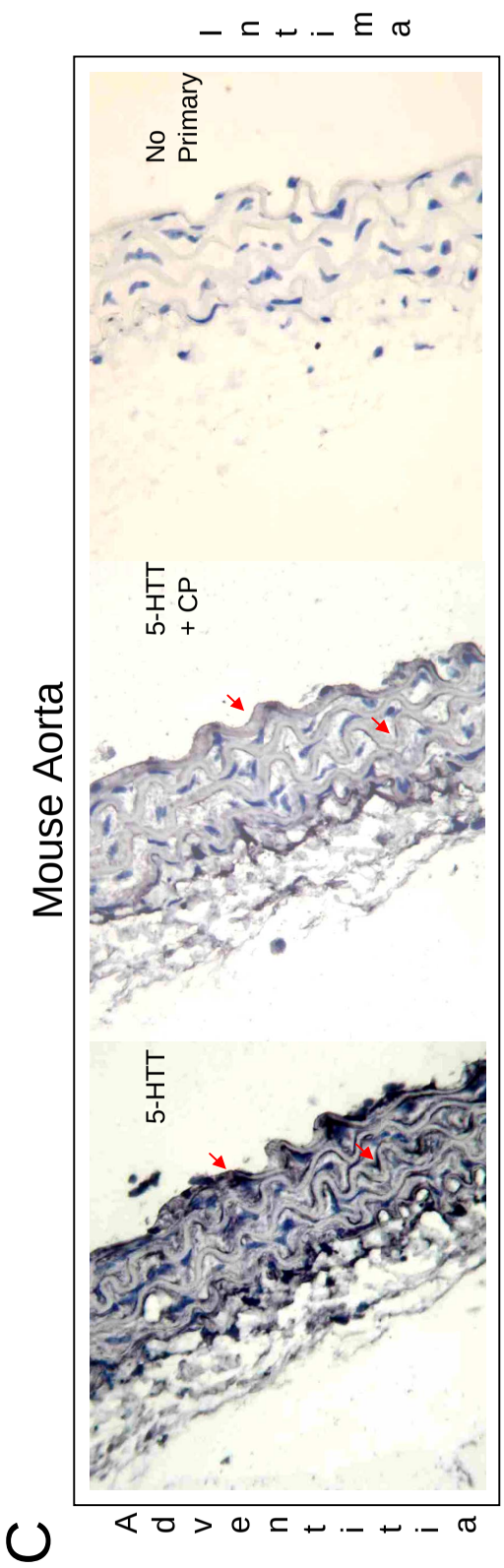


Figure 1

JPET Fast Forward. Published on May 18, 2005 as DOI: 10.1124/jpet.105.087080
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



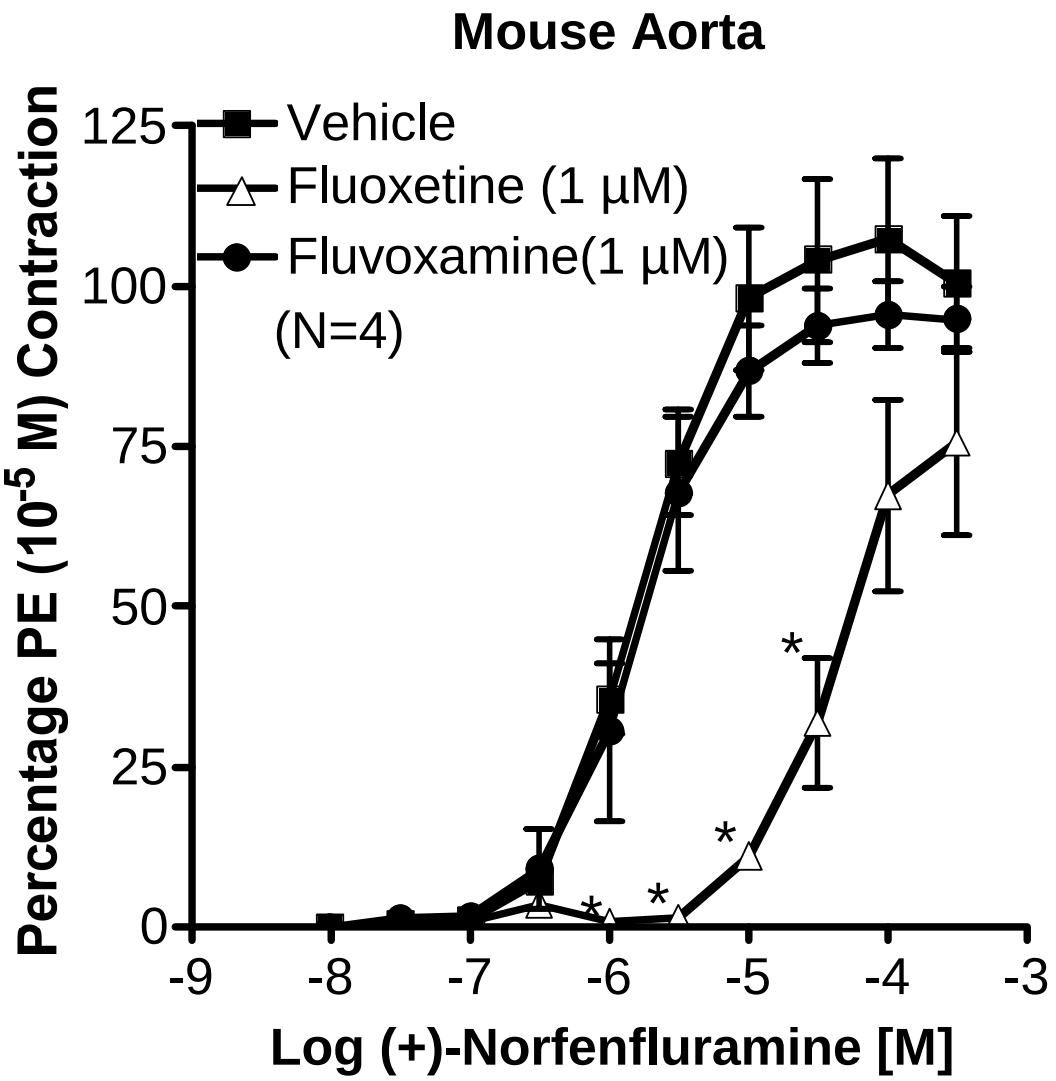


Figure 2

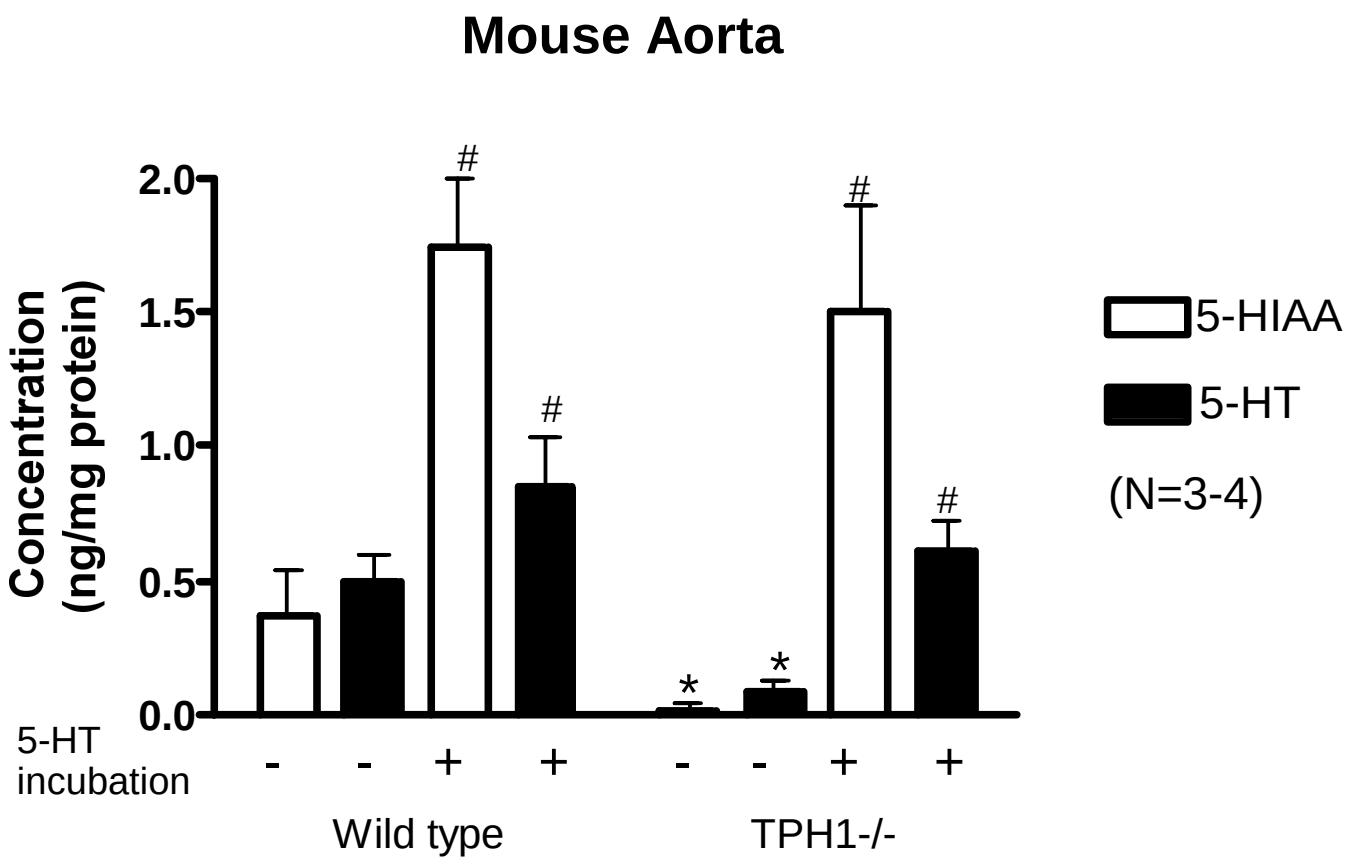


Figure 3

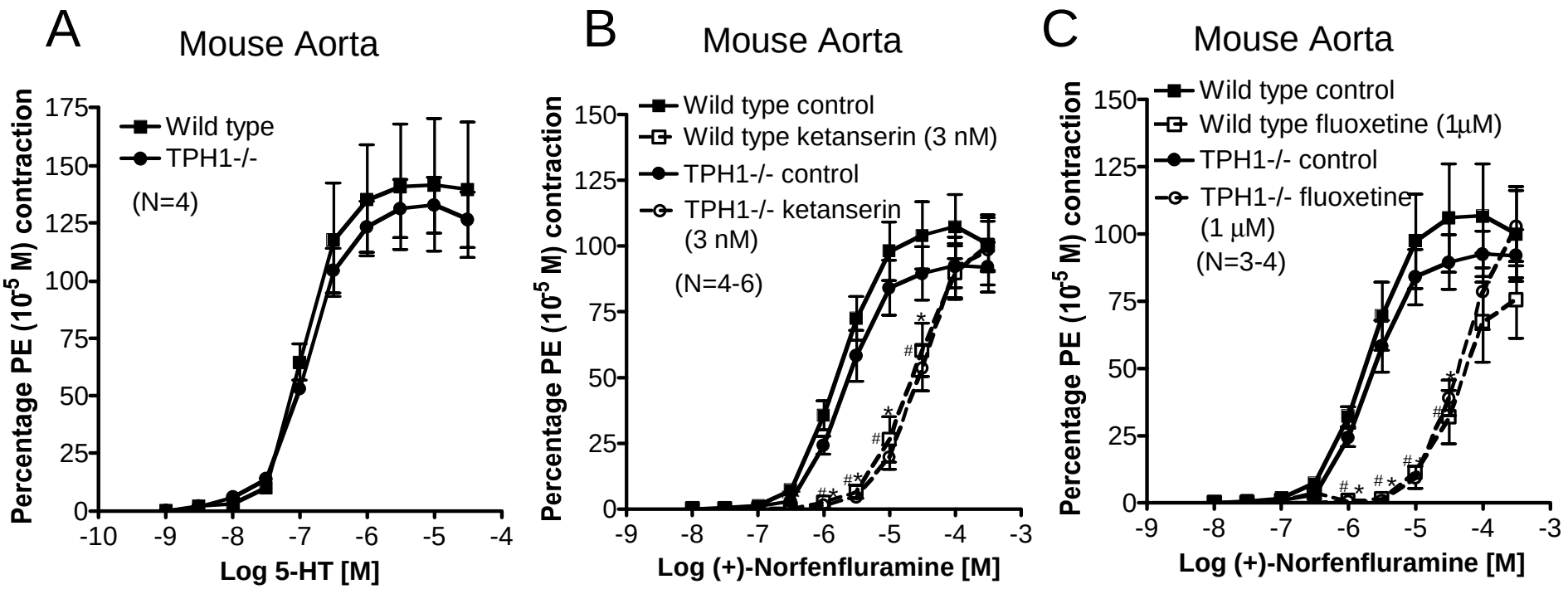


Figure 4

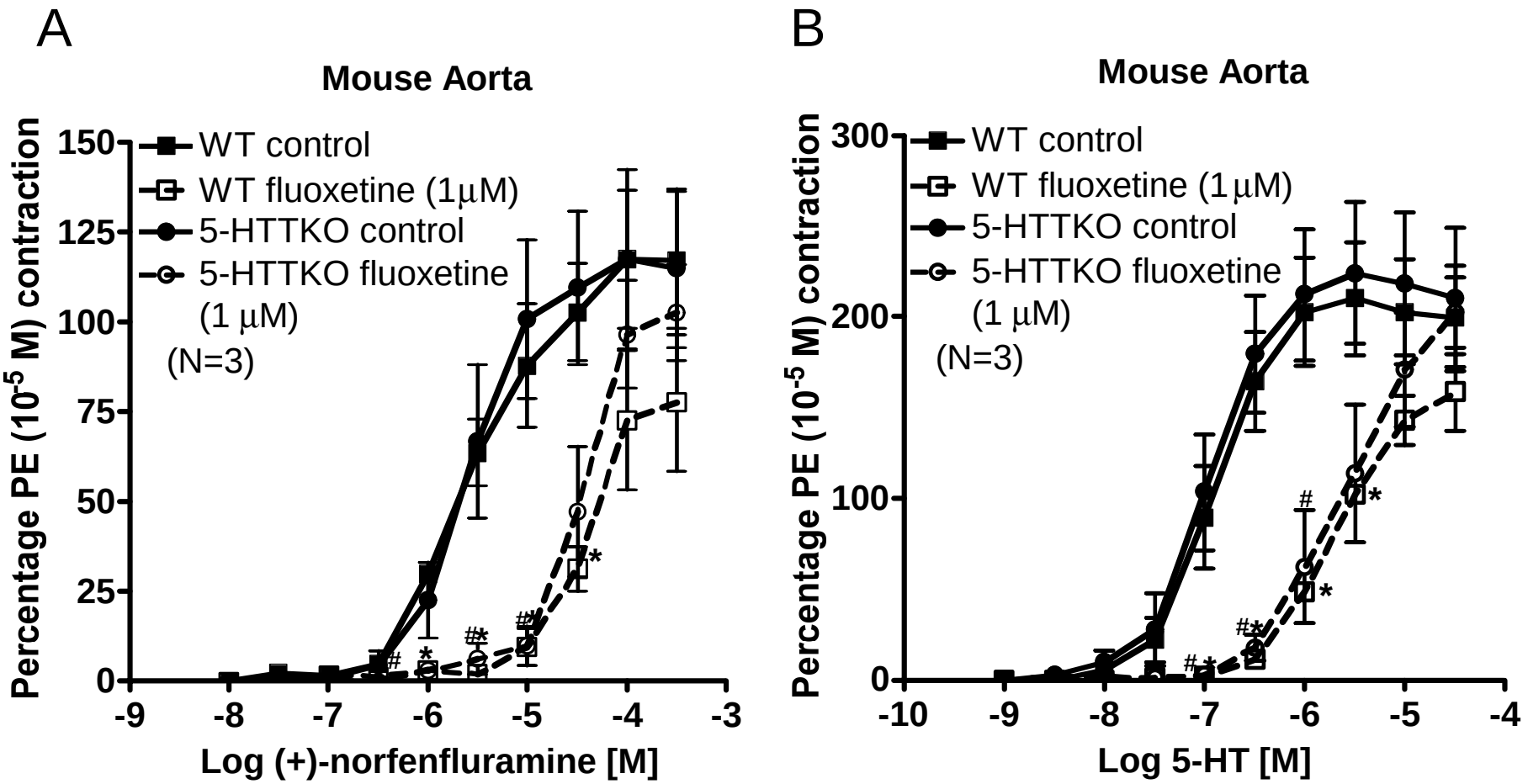


Figure 5

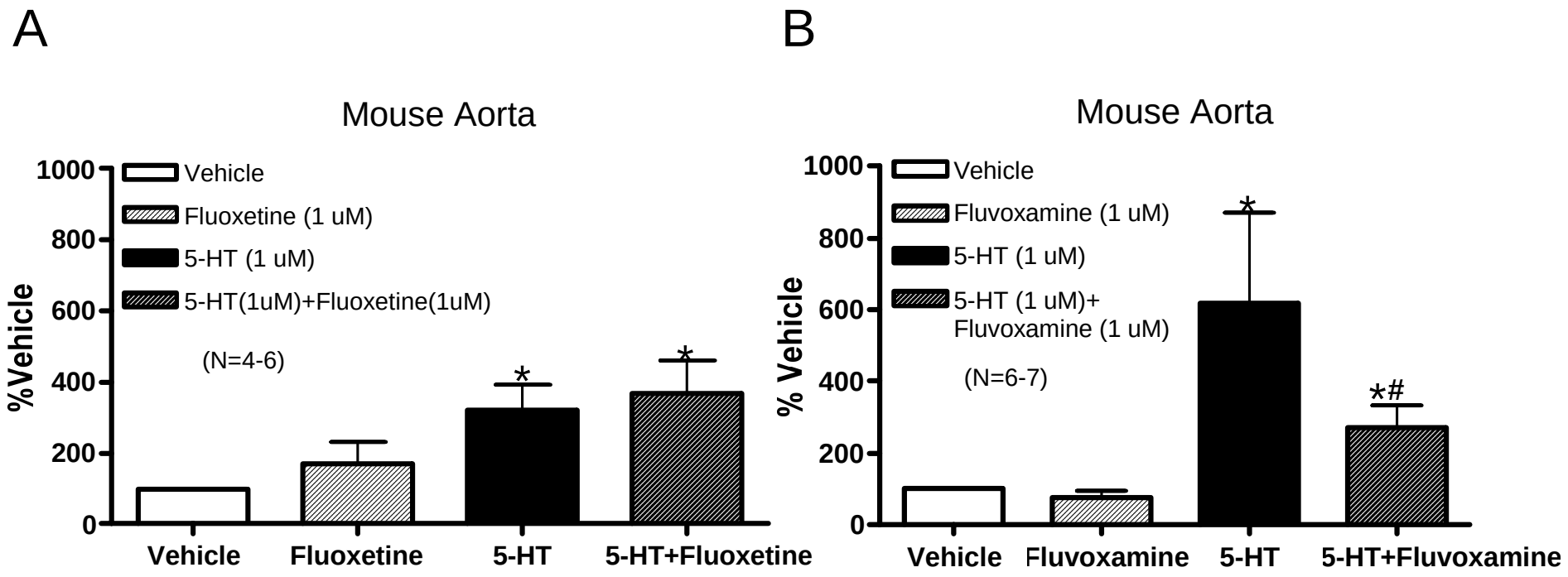


Figure 6