Amphetamine Analogs Increase Plasma Serotonin: Implications for Cardiac and Pulmonary Disease

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Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine or serotonin; ANOVA, analysis of variance; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; MAO, monoamine oxidase; MDMA, (±)-3,4-methylenedioxymethamphetamine; PPH, primary pulmonary hypertension; SERT, serotonin transporter; VHD, valvular heart disease

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ABSTRACT

Elevations in plasma serotonin (5-HT) have been implicated in the pathogenesis of cardiac and pulmonary disease. Normally, plasma 5-HT concentrations are kept low by transporter-mediated uptake of 5-HT into platelets and by metabolism to 5-hydroxyindoleacetic acid (5-HIAA). Many abused drugs (e.g., substituted amphetamines) and prescribed medications (e.g., fluoxetine) target 5-HT transporters, and could thereby influence circulating 5-HT. We evaluated the effects of amphetamines analogs [(±)-fenfluramine, (±)-MDMA, (+)-methamphetamine, (+)-amphetamine, phentermine] on extracellular levels (i.e., plasma levels) of 5-HT and 5-HIAA in blood from catheterized rats. Effects of the 5-HT uptake blocker fluoxetine were examined for comparison. Drugs were tested in vivo and in vitro; plasma indoles were measured using a novel microdialysis method in whole blood. We found that baseline dialysate levels of 5-HT are ~0.22 nM, and amphetamine analogs evoke large dose-dependent increases in plasma 5-HT ranging from 4-20 nM. The ability of drugs to elevate plasma 5-HT is positively correlated with their potency as 5-HT transporter substrates. Fluoxetine produced small, but significant, increases in plasma 5-HT. While the drug-evoked 5-HT concentrations are below the mM levels required for contraction of pulmonary arteries, they approach concentrations reported to stimulate mitogenesis in pulmonary artery smooth muscle cells. Additional studies are needed to determine the effects of chronic administration of amphetamines on circulating 5-HT.
INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an endogenous bioactive compound that is widely distributed in neurons, mast cells, enterochromaffin cells and blood platelets (Cooper et al., 2003; Gershon, 2004). Under normal physiological conditions, plasma 5-HT levels are kept exquisitely low (i.e., < 1 nM) due to transporter-mediated uptake of 5-HT into blood platelets, and via metabolism of 5-HT to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO). Included among the many physiological effects of 5-HT are mitogenesis and vasoconstriction. Accordingly, altered regulation of 5-HT levels in blood has been implicated in the pathogenesis of cardiac valvular heart disease (VHD) (Robiolio et al., 1995) and primary pulmonary hypertension (PPH) (MacLean et al., 2000). Many substituted amphetamine analogs (e.g., fenfluramine and aminorex) are substrates for 5-HT transporters (SERT) and release 5-HT from neurons via reversal of SERT (Rothman et al., 1999). One hypothesis to explain the ability of these agents to increase the risk of developing PPH is that they increase plasma 5-HT by stimulating 5-HT release from platelets (i.e., “the 5-HT hypothesis” of PPH) (MacLean et al., 2000). Others have invoked the same mechanism to explain fenfluramine-induced VHD (Fishman, 1999), although a more likely mechanism in this case involves activation of 5-HT$_2$B receptors by the N-deethylated metabolite of fenfluramine, norfenfluramine (Rothman et al., 2000).

Despite the importance of the 5-HT hypothesis to current dogma regarding the etiology of drug-induced PPH and VHD, the effects of anorectic agents on plasma 5-HT have received little attention. The paucity of data in this regard could be related to the fact that measuring plasma 5-HT is technically challenging. Given that basal 5-HT levels are quite low and that over
99% of blood 5-HT is stored in platelets, even minor disturbance of platelets during sample handling will cause large artificial increases in plasma 5-HT concentrations.

Studies conducted in the 1990s indicate that acute administration of (+)-fenfluramine does not increase plasma 5-HT in rats (Martin and Artigas, 1992), and chronic administration of fenfluramine lowers blood 5-HT in humans (see Rothman et al., 2000 and references therein). In the present study, we developed a novel microdialysis method to assess the effects of amphetamine analogs on plasma levels of 5-HT in whole blood samples obtained from conscious catheterized rats. The results show that amphetamine analogs produce significant dose-dependent increases in plasma 5-HT, and this effect is proportional to drug potency as SERT substrates. 5-HT uptake inhibitors, such as fluoxetine, also can increase plasma 5-HT but to a lesser extent. The physiological significance of these findings is discussed.
METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 350-450 g were singly housed with food and water freely available. Rats were maintained in facilities accredited by the American Association of the Accreditation of Laboratory Animal Care, and procedures were carried out in accordance with the Animal Care and Use Committee of the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP).

Drugs and Reagents

(±)-Fenfluramine HCl (fenfluramine, FW 267.7), (±)-3,4-methylenedioxymethamphetamine HCl (MDMA, FW 229.7), (+)-methamphetamine HCl (methamphetamine, FW 185.7), (+)-amphetamine sulfate (amphetamine, FW 368.5), phentermine HCl (phentermine, FW 185.7) and pentobarbital sodium were obtained from the NIDA, IRP Pharmacy. Fluoxetine HCl (fluoxetine, FW 345.8) was purchased from Spectrum Chemical Mfg. Company (New Brunswick, NJ). Monochloroacetic acid was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ) and all other reagents were obtained from Sigma Chemical Company (St. Louis, MO). Drug solutions for the in vivo and in vitro studies were prepared in saline immediately before use, and doses are expressed as the salt.

Surgical Procedures

Rats received sodium pentobarbital (60 mg/kg, i.p.) for surgical anesthesia. Indwelling jugular catheters, made of Silastic Medical Grade tubing (Dow Corning, Midland, MI), were implanted
into the right jugular vein and advanced to the atrium as described (Baumann et al., 2001). Rats were allowed to recover for 7-10 days post-operatively.

**In Vivo Drug Administration**

Between 8-9 AM, rats were moved into the testing room and allowed to acclimate to the surroundings for 1 h. Extension tubes were attached to catheters, and 0.5 ml of heparin flush (48 IU/ml in saline) was injected. Blood samples (0.3 ml) were withdrawn into 1 ml syringes and gently transferred into 300 µl polypropylene vials that were chilled on crushed ice. Vials contained 20 µl of heparin (1000 IU/ml in saline) as an anticoagulant. Heparin flush was injected into the rats after each sample to maintain volume homeostasis. A 4 x 0.6 mm dialysis probe (MAB 6, SciPro, Inc., Sanborn, NY) was immediately immersed in the chilled blood sample. Ringers’ solution containing 150.0 mM Na⁺, 3.0 mM K⁺, 1.4 mM Ca++, 0.8 mM Mg++, 1.0 nM P, and 155 mM Cl⁻ was pumped through the probe at 1.7 µl/min, and each blood sample was dialyzed for 15 min to generate a single dialysate sample. Serial blood samples were collected and dialyzed every 15 minutes. Dialysates were assayed for 5-HT and 5-HIAA using high performance liquid chromatography with electrochemical detection (HPLC-ECD) as described below. After 3-4 baseline samples were obtained and dialyzed, drug treatments were administered through i.v. catheters. Fenfluramine, MDMA, methamphetamine, amphetamine and fluoxetine were dissolved in saline and administered as i.v. bolus injections of 0.3 and 1.0 mg/kg. Phentermine was dissolved in saline and injected i.v. at doses of 1.0 and 3.0 mg/kg. Blood samples were collected at 15 min intervals for 90 min post-injection. Probe recoveries were performed before and after blood sampling using a 10 pg 5-HT standard prepared in Ringers’ solution.
In Vitro Drug Administration

Between 8-9 AM, rats were moved into the testing room and allowed to acclimate to the surroundings for 1 h. Extension tubes were attached to catheters, and 0.5 ml of heparin flush (48 IU/ml in saline) was injected as noted above. In these experiments, serial blood samples were withdrawn from untreated donor rats and transferred into 300 µL polypropylene tubes which were kept at 25°C. These tubes were prefilled with 20 µL of heparin (1000 IU/ml). Test drugs or vehicle were added directly to blood samples in 10 µL volumes to yield final concentrations of 0.3, 1, 3 or 33 µM. Microdialysis probes were placed into the blood samples and dialysate efflux was collected for 15 min and assayed for 5-HT and 5-HIAA using HPLC-ECD. Each blood sample was dialyzed for 15 min to generate a single dialysate sample. Two baseline samples were collected before addition of test drugs to subsequent samples. Probe recoveries were performed before and after blood sampling using a 10 pg 5-HT standard.

HPLC-ECD Analysis of 5-HT and 5-HIAA

Aliquots of the dialysate (5 µl) were injected onto a microbore HPLC column (Unijet, 100 x 1 mm, 5 µM ODS, Bioanalytical Systems, Inc, West Lafayette, IN) that was coupled to an amperometric detector (Model LC-4C, BAS, Inc.). A glassy carbon electrode was set at a potential of +650 mV relative to Ag/AgCl reference. Mobile phase consisted of 180 µM Na₂EDTA, 150 mM monochloroacetic acid, 125 mM NaOH, and 690 µM sodium octanesulfonic acid, with 7.5 % MeOH and 7.5% CH₃CN per liter of water (final pH= 3.15). Mobile phase was pumped through the column at 60 µl/min (260D, syringe pump, Teledyne ISCO, Lincoln, NE). Chromatographic data were acquired on-line and exported to a Millennium software system.
(Waters Associates, Milford, MA) for peak amplification, integration and analysis. The concentration of 5-HT and 5-HIAA in dialysate samples was compared to known standards, and the lower limit of detection was ~ 0.05 pg/5 μL (0.047 nM) for both indoles.

Statistical Analyses

In all studies, the first 2 dialysate samples collected before any treatment were considered baseline samples. 5-HT and 5-HIAA measures are mean±SEM expressed as pg per 5 μL sample. For in vivo experiments, data were evaluated by 2-way ANOVA (drug treatment x time) and 1-way ANOVA (at each time point). For in vitro experiments, data were evaluated by 1-way ANOVA (drug dose). When significant F values were obtained, Newman-Keul’s post-hoc tests were performed to compare group means. For data correlations, peak drug effects measured in vivo after 1.0 mg/kg were compared to peak drug effects measured in vitro after 3 μM and to drug EC₅₀ values for [³H]5-HT release from synaptosomes (Rothman et al, 2001). A value of P<0.05 was considered as the minimum criterion for statistical significance.
RESULTS

Baseline 5-HT Levels in Plasma

For all animals used in this study (N=172 rats), the mean basal concentration of dialysate 5-HT in blood was 0.23±0.01 pg/μL (i.e., 0.22±0.01 nM). Baseline dialysate 5-HT levels differed slightly depending upon experimental conditions. In the *in vivo* drug administration experiments (N=120 rats), blood samples were maintained on ice to reduce SERT-mediated ‘leak’ of 5-HT from platelets, and basal plasma 5-HT was 0.20±0.01 pg/μL. In the *in vitro* drug experiments (N=52 rats), blood samples were kept at room temperature to optimize SERT function, and this resulted in a slightly higher basal level of plasma 5-HT, 0.29±0.02 pg/μL. Baseline 5-HT did not differ significantly between the various treatment groups within the *in vivo* and *in vitro* conditions. It should be noted that microdialysis probes had *in vitro* recovery rates of approximately 25% when tested in a physiological salt solution, and this value did not vary significantly before, during or after experiments where probes were immersed in sequential blood samples. Preliminary studies have shown that *in vitro* probe recoveries determined in artificial salt solutions do not necessarily reflect probe recovery characteristics in complex biological matrices. Thus, we did not ‘correct’ 5-HT or 5-HIAA values for probe recovery. In all cases, separate saline control groups were tested in parallel with each drug treatment condition.

*In Vivo* Drug Administration Experiments

Figure 1 depicts the effects of fenfluramine, MDMA, methamphetamine, amphetamine, phentermine and fluoxetine on dialysate 5-HT levels in whole blood, when drugs were
administered via i.v. catheters in vivo. Fenfluramine significantly increased dialysate 5-HT [F(2,120)= 56.55; p<0.0001] and this stimulatory action was dose-dependent. Fenfluramine elevated extracellular 5-HT to 15- and 19-fold after 0.3 and 1.0 mg/kg, respectively. MDMA also significantly increased 5-HT levels [F(2,120)= 64.22; p< 0.0001], with 12- and 21-fold elevations at the 0.3 and 1.0 mg/kg doses. Methamphetamine significantly increased plasma 5-HT [F(2,120)=29.75; p<0.0001] but to a lesser extent than fenfluramine, with 7- and 12-fold elevations at 0.3 and 1.0 mg/kg doses. Amphetamine had weak effects on 5-HT levels [F(2,120)= 3.64; p= 0.03] and only the 1mg/kg dose caused a significant 5-fold rise.

Phentermine had a very weak effect on 5-HT and post-hoc tests failed to demonstrate any significant effects of the 1.0 and 3.0 mg/kg doses, possibly due to variability in the saline-injected control group. The 5-HT reuptake inhibitor, fluoxetine, produced a modest increase in plasma 5-HT [F(2,120)=49.16, p<0.0001] with 0.3 and 1.0 mg/kg doses increasing 5-HT levels 4- and 7-fold. Interestingly, none of the in vivo treatments affected plasma 5-HIAA levels (data not shown). Fenfluramine, MDMA, and methamphetamine produced transient increases in plasma 5-HT that had mostly returned to baseline values after 90 min.

Some investigators (Ulus et al., 2000) have proposed that pharmacological doses of phentermine and other amphetamine analogs will block MAO activity in vivo, an effect which can be detected as decreased 5-HIAA levels. Others have suggested that co-administration of phentermine with fenfluramine would enhance fenfluramine-induced increases in plasma 5-HT (Ulus et al., 2000; Fishman, 1999). To address these hypotheses, we compared the effects of phentermine and fenfluramine, alone and in combination, at 1.0 mg/kg doses. As shown in Fig. 2, no treatment altered plasma 5-HIAA, and co-administration of phentermine plus fenfluramine did not enhance plasma 5-HT higher than fenfluramine alone.
**In Vitro Drug Administration Experiments**

Fig. 3 shows the effects of fenfluramine, MDMA, methamphetamine, amphetamine, phentermine and fluoxetine on dialysate 5-HT levels in blood, when drugs were administered directly into blood samples *in vitro*. Fenfluramine significantly increased plasma 5-HT in a dose-dependent manner \[F(3,23)=64.54, p<0.0001\], producing 16- and 91-fold elevations when administered at concentrations of 3 and 33 \(\mu\)M, respectively. MDMA had similar effects \[F(3,23)=16.15, p<0.0001\] and increased dialysate 5-HT to 8- and 42-fold above baseline at 3 and 33 \(\mu\)M doses. Methamphetamine \[F(3,23)=96.19, p<0.0001\] and amphetamine \[F(3,23)=50.03, p<0.0001\] were somewhat less efficacious than MDMA, significantly increasing 5-HT by 4- and 44-fold at the 3 and 33 \(\mu\)M doses, respectively. Phentermine significantly increased 5-HT levels only at the 33 \(\mu\)M dose \[F(3,23)=9.71, p<0.0004\]. Fluoxetine dose-dependently increased plasma 5-HT \[F(3,23)=17.76, p<0.0001\] to 3-, 14- and 22-fold above baseline at 0.3 , 3 and 33 \(\mu\)M, respectively.

The data in the top panel of Fig. 4 show that the ability of amphetamine analogs to increase plasma 5-HT *in vitro* at a 3 \(\mu\)M dose is significantly correlated with the increase in 5-HT levels produced after *in vivo* administration of 1.0 mg/kg (P<0.04). This finding suggests that these drugs are increasing extracellular 5-HT in blood by a similar mechanism under *in vitro* and *in vivo* conditions. We previously reported the EC\(_{50}\) values for drugs releasing \[^3\text{H}\]5-HT from synaptosomes, which is a measure of the potency of these compounds as substrates for SERT (Rothman et al., 2001). The bottom panel of Fig. 4 demonstrates that the calculated EC\(_{50}\) values for test drugs to release \[^3\text{H}\]5-HT from synaptosomes is highly correlated with the ability of the same agents to increase plasma 5-HT *in vivo* (P<0.01). Taken together, these correlative...
relationships suggest that amphetamine analogs increase plasma 5-HT in whole blood by a process involving SERT proteins, possibly those found on platelets.

Studies in nervous tissue have shown that uptake inhibitors can attenuate the ability of transporter substrates to release 5-HT via carrier-mediated exchange (Baumann e al., 2001; Rothman et al., 2001). To test whether uptake blockers could affect drug-induced 5-HT efflux in blood samples, we co-administered fluoxetine and MDMA in blood samples in vitro and performed microdialysis. The top panel of Fig. 5 demonstrates that low-dose fluoxetine (i.e., 0.3 µM) did not block the rise in plasma 5-HT produced by low-dose MDMA (i.e., 1.0 µM). This type of experiment is complicated by the fact that fluoxetine pretreatment alone causes increases in plasma 5-HT that are comparable to the effects of low-dose MDMA. However, as reported in the bottom panel of Fig. 5, a higher dose of fluoxetine (i.e., 3 µM) significantly reduced the large increase in plasma 5-HT produced by 33 µM MDMA by 50% [F(3,39)=70.61, p<0.0001].
DISCUSSION

Fenfluramine, MDMA, methamphetamine and amphetamine are known to be substrates for SERT proteins and to release 5-HT from neurons in the brain (Berger et al., 1992; Crespi et al., 1997; Rothman et al., 2001). The anorectic medication, fenfluramine, is associated with the occurrence of PPH and VHD, and was withdrawn from the marketplace in 1997. Determining the mechanism(s) whereby fenfluramine increases the risk of developing VHD and PPH is important to understand, so that newly discovered serotonergic medications will not produce these serious adverse effects (Rothman and Baumann, 2003). Many investigators have theorized that fenfluramine increases the risk of PPH and VHD by elevating plasma 5-HT levels in blood, consistent with its known 5-HT-releasing properties in nervous tissue (Connolly et al., 1997; Fishman, 1999; MacLean et al., 2000). Studies conducted in the 1990s provide little support for this hypothesis. For example, Martin and Artigas (1992) reported that acute administration of (+)-fenfluramine does not increase plasma 5-HT in rats. Moreover, chronic administration of fenfluramine lowers blood 5-HT in humans (see Rothman et al., 2000 and references therein). Given the importance of the 5-HT hypothesis to current dogma regarding fenfluramine-associated PPH and VHD, we decided to directly investigate this issue by using a novel microdialysis method to assess the effects of amphetamine analogs on plasma levels of 5-HT in conscious rats. In addition to fenfluramine, we assessed the actions of other substituted amphetamines on plasma 5-HT, both therapeutic agents such as amphetamine, and drugs of abuse such as MDMA and methamphetamine.

Measuring plasma 5-HT is technically challenging. Given that over 99% of blood 5-HT is stored in platelets, and platelets are very fragile, even minor disturbance or damage to platelets
during sample handling will cause large artificial increases in plasma 5-HT. The method we developed to measure dialysate 5-HT levels ex vivo minimizes trauma to platelets and permits an accurate determination of plasma 5-HT. The mean baseline level of dialysate 5-HT in blood collected from rats in these studies (N=172) was 0.22±0.01 nM. Because *in vitro* probe recoveries averaged about 25%, the ‘actual’ corrected baseline level of plasma 5-HT in our experiments is likely in the range of 0.88 nM. This level is comparable to plasma 5-HT levels in humans, which are reportedly in the sub-nM range (Herve et al., 1995).

With the exception of phentermine, all test drugs produced dose-dependent increases in plasma 5-HT when administered *in vivo* (see Fig. 1). These findings differ from those of Martin and Artigas (1992), who did not observe elevations in plasma 5-HT after administration of (+)-fenfluramine (2.5 mg/kg) to rats. The reason for the discrepancy between our results and those of others is not known, but could be related to differences in blood sampling procedures. Specifically, we obtained blood samples within 15 min of fenfluramine administration whereas Martin and Artigas sampled 60 min after injection, possibly missing the peak effect of the drug on circulating 5-HT.

The ability of amphetamine analogs to increase plasma 5-HT *in vivo* is correlated with their capacity to increase plasma 5-HT *in vitro*, and with their potency at releasing [*3H*]5-HT from synaptosomes (see Fig. 4). Since platelets and neurons express the same SERT protein (Lesch et al., 1993), the potency of agents at releasing [*3H*]5-HT from platelets and from synaptosomes should be similar. Indeed, the EC$_{50}$ value reported by Schuldiner et al. (1993) for fenfluramine-induced release of [*3H*]-5HT from platelets (170 nM) is similar to the value we reported for fenfluramine in rat brain synaptosomes (79 nM) (Rothman et al., 2001). These observations support the notion that amphetamine analogs act as substrates for platelet SERT
proteins, thereby explaining their ability to increase plasma 5-HT. This interpretation is further supported by the finding that fluoxetine could partially reverse the ability of high-dose MDMA to release 5-HT in vitro (see Fig. 5). In this experiment, we believe that fluoxetine prevents MDMA from entering platelets by blocking SERT sites, thus preventing massive release of 5-HT via disruption of storage vesicles (Schuldiner et al., 1993). Both methamphetamine and amphetamine release 5-HT from synaptosomes with low potency (~ 1 µM), but methamphetamine is much more potent at increasing extracellular 5-HT in brain via a SERT-related mechanism (Rothman et al., 2005; Rothman and Baumann, 2003). Although we have no explanation for this peculiar phenomenon, it also occurs in blood since methamphetamine, but not amphetamine, increases plasma 5-HT at the 0.3 mg/kg dose. As noted above, the 40-fold increase in plasma 5-HT produced by 33 µM amphetamine in vitro likely results from disruption of 5-HT storage vesicles in the platelets.

Steady state plasma 5-HT levels are maintained in the sub-nM range in part by SERT-mediated uptake of 5-HT into platelets. Thus, it is not surprising that the 5-HT uptake inhibitor fluoxetine also increases plasma 5-HT in vivo and in vitro. The stimulatory effect of fluoxetine is much smaller in magnitude than the effects of amphetamines, suggesting that uptake blockers produce minor changes in plasma 5-HT when compared to SERT substrates. It should be noted that fluoxetine, unlike SERT substrates, cannot be removed from the plasma by translocation into cells that express SERT, and this could prolong the actions of fluoxetine and its bioactive metabolite, norfluoxetine. Since the ability of fluoxetine to increase plasma 5-HT arises from its inhibition of SERT, other serotonin selective reuptake inhibitors (SSRIs) might cause small and transient increases in plasma 5-HT. It is unlikely that SSRI-induced increases in plasma 5-HT contribute to the antidepressant effects of this class of medication. However, it is tempting to
speculate that SSRI-induced increases in plasma 5-HT contribute to the ability of SSRIs to increase ejaculatory threshold (de Jong et al., 2006).

Our findings provide at least part confirmation the 5-HT hypothesis – that fenfluramine can increase plasma 5-HT. However, it is possible that the ability of fenfluramine and other SERT substrates to increase plasma 5-HT might be different after chronic administration. In this instance, platelet 5-HT is markedly reduced (Raleigh et al., 1986; Celada et al., 1994), and it is not yet known if plasma 5-HT is reduced under these circumstances or if the ability of fenfluramine to increase plasma 5-HT will be attenuated. In the case of chronic treatment with SERT inhibitors, both platelet and plasma 5-HT are markedly reduced (Celada et al., 1992).

Regarding PPH, another factor to consider is whether the maximal drug-induced plasma 5-HT concentrations approach those needed to contract pulmonary arteries or to stimulate mitogenesis in pulmonary artery endothelium or smooth muscles. The maximal concentration of drug-induced dialysate 5-HT was ~ 5 nM when high i.v. drug doses were administered; correcting for probe recovery estimates, this value is increased to ~ 20 nM. Serotonin contracts human pulmonary arteries with EC_{50} values that range from about 100 nM (Morecroft et al., 1999) to the \mu M range (Cortijo et al., 1997). Thus, it would seem unlikely that fenfluramine and other SERT substrates could increase plasma 5-HT to a concentration that would directly contract pulmonary arteries.

Recent studies indicate that SERT plays a key role in mitogenic effect of 5-HT on pulmonary artery smooth muscle cells, and this effect can be prevented by SERT inhibitors fluoxetine and paroxetine but not by the 5-HT_{2A} receptor antagonist ketanserin (Eddahibi et al., 2002). The threshold concentration for 5-HT-stimulated mitogenic responses in cultured human pulmonary artery smooth muscle cells is about 10 nM (Eddahibi et al., 2001; Marcos et al.,
2003), although higher 5-HT (~100 nM) concentrations are needed to stimulate mitogenic responses in rat pulmonary artery smooth muscle cells (Pitt et al., 1994; Eddahibi et al., 1999). It seems possible that high doses of fenfluramine, MDMA and methamphetamine could transiently produce plasma 5-HT concentrations sufficient to stimulate mitogenic responses in pulmonary smooth muscle cells. Consequently, these drugs could increase the risk of developing PPH in susceptible individuals, should the exposure to higher than normal plasma 5-HT continue long enough.

Regarding VHD, it is well established that 5-HT can induce mitogenic responses in human and animal heart interstitial cells in vitro (Rajamannan et al., 2001; Jian et al., 2002; Setola et al., 2003). Although mitogenic responses have been observed with 5-HT concentrations as low as 10 nM (Hafizi et al., 2000; Rajamannan et al., 2001), other studies report that 5-HT concentrations in the µM range are required to stimulate mitogenic responses (Jian et al., 2002; Setola et al., 2003). Importantly, cells must be exposed to 5-HT for 24-48 h to produce the mitogenic response, a period of time far greater than the 60-90 min of elevated plasma 5-HT produced by administration of fenfluramine and the other substituted amphetamines. Thus, it seems unlikely that a single i.v. dose of fenfluramine, MDMA or methamphetamine could transiently produce high enough plasma 5-HT concentrations to stimulate mitogenic responses in cardiac valvular cells. This interpretation of our data is supported by the fact that high 5-HT concentrations (> 500 nM) are required to produce valvulopathy in carcinoid syndrome (Robiolio et al., 1995). Modest 2-3-fold elevations of plasma 5-HT, such as those occurring with the prescribed use of lithium (Artigas et al., 1989) and MAO inhibitors (Celada et al., 1992) are not associated with VHD. Our previous findings indicated that medications known to cause VHD (fenfluramine, ergotamine, methysergide)
produce metabolites which are 5-HT$_{2B}$ receptor agonists (Rothman et al. 2000; Setola et al., 2003). More recently, two medications used to treat Parkinson’s disease, pergolide and cabergoline, were identified as 5-HT$_{2B}$ agonists that also cause VHD (for review see: Setola and Roth, 2005). Collectively the data indicate that fenfluramine produces VHD through the activation of cardiac valvular 5-HT$_{2B}$ receptors by its metabolite, norfenfluramine, and not from elevation of plasma 5-HT. On the other hand, the translocation of 5-HT transporter substrates into cells in exchange for endogenous 5-HT, and the subsequent ‘trapping’ of these agents inside of cells, could contribute to the mechanism of toxicity (Rothman et al., 1999).

Our experiments provided the unique opportunity to examine other hypotheses concerning the pathogenesis of fenfluramine-associated VHD. Ulus et al. (2000) suggested that phentermine and other amphetamines block MAO activity in vivo, and this action could contribute to the development of VHD when phentermine and fenfluramine are administered together. MAO is an enzyme that metabolizes 5-HT to 5-HIAA. Inhibition of MAO produces measurable decreases in plasma 5-HIAA. Several types of evidence obtained in rat nervous tissue suggest that phentermine and fenfluramine do not affect MAO (Baumann et al., 2000; Kilpatrick et al., 2001), and the present data confirm that high i.v. doses of these drugs do not affect plasma 5-HIAA (see Fig. 2), consistent with data from non-human primates (Alexander et al., 2005). Other investigators proposed that phentermine would enhance fenfluramine-induced increases in plasma 5-HT (Fishman, 1999; Ulus et al., 2000). Our data clearly show that phentermine does not enhance the rise in plasma 5-HT produced by fenfluramine.

In summary, contrary to previously published data (Martin and Artigas, 1992), we demonstrate that fenfluramine and other SERT substrates produce significant dose-dependent increases in plasma 5-HT when administered under in vivo and in vitro conditions. This effect
most likely involves SERT-mediated exchange of drug molecules for platelet 5-HT. Agents with less potent SERT substrate activity, such as amphetamine and phentermine, are considerably less effective in elevating plasma 5-HT when compared to fenfluramine, MDMA and methamphetamine. The fact that MDMA and methamphetamine increase plasma 5-HT to concentrations that stimulate mitogenic responses in pulmonary artery smooth muscle cells suggests that this effect may contribute to the cardiovascular toxicities associated with illicit use of these drugs. Further research will be needed to elucidate the physiological significance of our findings.
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REFERENCES


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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. *In vivo* effects of amphetamine analogs and fluoxetine on dialysate 5-HT levels measured in blood from conscious rats. Drugs were dissolved in sterile saline and administered i.v. at 0 min. Serial blood samples were withdrawn at 15 min intervals and immediately dialyzed as described in Methods. Data are mean±SEM for N=6 rats/group. * = P<0.05 compared to saline controls at corresponding time points.

Figure 2. *In vivo* effects of fenfluramine (fen) and phentermine (phen) on dialysate 5-HT and 5-HIAA levels measured in blood from conscious rats. Drugs were dissolved in sterile saline and administered by the i.v. route. Fen or phen was given at a dose of 1.0 mg/kg, whereas fen + phen was administered as a mixture to yield a final concentration of 1.0 mg/kg for each drug. Serial blood samples were withdrawn at 15 min intervals and were immediately dialyzed. Data show peak effects determined 15 min post-injection and are mean±SEM for N=6 rats/group. * = P<0.05 with respect to saline-treated control.

Figure 3. *In vitro* effects of amphetamine analogs and fluoxetine on dialysate 5-HT levels measured in blood from conscious rats. Drugs were added directly to blood samples obtained from untreated donor rats, to yield final concentrations shown. Blood samples were immediately dialyzed as described in Methods. Data show peak effects measured 15 min after addition of drugs and are mean±SEM for N=6 rats/group. * = P<0.05 compared to saline control.
Figure 4. Drug-induced elevations of plasma 5-HT in vivo are correlated with increases in plasma 5-HT measured in vitro (top panel) and EC$_{50}$ values for [³H]5-HT release measured in synaptosomes (bottom panel). For the top panel, the % increase in plasma 5-HT induced by 1 mg/kg in vivo was plotted against % increase induced by 3 µM in vitro for each amphetamine tested. In the bottom panel, the % increase in plasma 5-HT induced by 1 mg/kg in vivo was plotted against the EC$_{50}$ for [³H]5-HT release from synaptosomes previously reported for each amphetamine tested. The in vivo and in vitro plasma 5-HT data are means for N=6 rats/group, whereas the EC$_{50}$ data are means for N=3 separate experiments.

Figure 5. In vitro effects of fluoxetine on MDMA-induced increases in dialysate 5-HT levels measured in blood from conscious rats. Drugs were added directly to blood samples obtained from untreated donor rats to yield final concentrations shown as µM equivalents. Blood samples were immediately dialyzed. Data show peak effects measured 15 min after addition of drugs and are mean±SEM for N=6-10 rats/group. * = P<0.05 compared to saline control; # = P<0.05 with respect to all other groups.
Figure 1

Fenfluramine

- saline
- 0.3 mg/kg
- 1 mg/kg

MDMA

- saline
- 0.3 mg/kg
- 1 mg/kg

Methamphetamine

- saline
- 0.3 mg/kg
- 1 mg/kg

Amphetamine

- saline
- 0.3 mg/kg
- 1 mg/kg

Phentermine

- saline
- 1 mg/kg
- 3 mg/kg

Fluoxetine

- saline
- 0.3 mg/kg
- 1 mg/kg
Figure 2

![Graph showing indole (pg/5µl) levels with conditions: saline, fen, phen, fen+phen. The graph compares 5-HT and 5-HIAA levels.](image-url)
Figure 3

- **Fenfluramine**: Shows a significant increase in 5-HT levels with concentrations of 3 and 33 µM.
- **MDMA**: Exhibits a concentration-dependent effect, with a significant increase at 33 µM.
- **Methamphetamine**: Demonstrates a similar pattern to Fenfluramine, with a significant rise at 33 µM.
- **Amphetamine**: Like MDMA, shows a significant increase at 33 µM.
- **Phentermine**: Also exhibits a concentration-dependent effect, with a significant rise at 33 µM.
- **Fluoxetine**: Shows a less pronounced effect but maintains a concentration-dependent trend.

*Note: The figure illustrates the effect of various concentrations on 5-HT levels with statistical significance indicated by *.
Figure 4

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