Increased release of serotonin from rat ileum due to dexfenfluramine

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Rezaie-Majd, Shahrzad, Jozef Murar, Daniel P. Nelson, Rosemary F. Kelly, Zhigang Hong, Irene M. Lang, Anthony Varghese, and E. Kenneth Weir. Increased release of serotonin from rat ileum due to dexfenfluramine. Am J Physiol Regul Integr Comp Physiol 287: R1209–R1213, 2004. First published July 8, 2004; doi:10.1152/ajpregu.00191.2004.—Plasma levels of serotonin are elevated in primary pulmonary hypertension even after bilateral lung transplantation, suggesting a possible etiologic role. Serotonin is released primarily from the small intestine. Anorectic agents, such as dexfenfluramine, which can cause pulmonary hypertension, are known to inhibit potassium channels in vascular smooth muscle cells. We examined the hypothesis that dexfenfluramine may stimulate release of serotonin from the ileum by inhibition of K+ channels. In an isolated loop of rat ileum perfused with a physiological salt solution, the administration of dexfenfluramine, its major metabolite D-norfenfluramine, the potassium channel blocker 4-aminopyridine (5 mM), and caffeine (30 mM) increased serotonin levels in the venous effluent. Potassium chloride (60 mM) tended to increase serotonin levels. In genetically susceptible individuals, dexfenfluramine may induce pulmonary hypertension by increasing cytosolic calcium in enterochromaffin cells of the small intestine, thus releasing serotonin and causing vasoconstriction. This work indicates that dexfenfluramine and its major metabolite D-norfenfluramine can increase serotonin release from the small intestine.

enterochromaffin; potassium channel; hypertension

ENTEROCHROMAFFIN (EC) cells of the intestinal tract are part of a diffuse neuroendocrine system. EC cells are responsible for 90% of the circulating plasma serotonin. Serotonin is known to cause vasoconstriction and cellular proliferation in pulmonary arteries (6a). Serotonin is elevated in the plasma of patients with primary pulmonary arterial hypertension and of patients with pulmonary hypertension associated with the use of anorectic agents (8, 12, 28). The recent discovery of the connection between primary pulmonary hypertension (PPH) and a mutation of the gene for the bone morphogenetic protein receptor type II (BMPR-II) partly explains the etiology and pathophysiology of PPH (19). This mutation has been identified as being involved in the development of over 50% of familial (5, 14) and 25% of sporadic forms of PPH (30). Because only a fraction of patients with a BMPR-II mutation develop PPH (16), a mechanism involving “a second insult” has been suggested. It is possible that in the presence of a BMPR-II gene mutation, different triggering mechanisms may be responsible for development of PPH. These mechanisms could involve increased plasma serotonin, inhibition of the serotonin transporter (SERT), activation of a serotonin receptor (5-HTR), inhibition of potassium (K+) current in pulmonary artery smooth muscle cells (PASMCs), or a reduction in endogenous nitric oxide. The case for serotonin as a triggering mechanism is based on the observation that plasma serotonin levels are elevated in patients with PPH and remain elevated even after bilateral lung transplantation (8) and on the report of the development of PPH in a patient with a familial platelet storage pool disease and high plasma serotonin (7). In addition, plasma serotonin levels are elevated in the fawn-hooded rat that develops spontaneous pulmonary hypertension.

The anorectic agents aminorex and dexfenfluramine are believed to cause reduced appetite by inducing release of serotonin in the central nervous system (29) and by inhibiting its reuptake through the serotonin transporter (24, 25), thus increasing interstitial concentrations of serotonin. Both of these agents have been associated with a marked increase in the incidence of pulmonary hypertension clinically and histologically indistinguishable from PPH (1). As both drugs have been withdrawn from the market, it is no longer possible to establish whether they increase plasma levels of serotonin in humans and prior data are conflicting. Recent data do indicate that increasing doses of dexfenfluramine given orally to dogs for extended periods cause increasing plasma levels of serotonin (10, 11). It has also been reported that serotonin metabolism is not decreased in pulmonary hypertension (12). If this were true in PPH, the observed high plasma levels of serotonin would suggest that production might be increased.

This study demonstrates that dexfenfluramine and its major metabolite D-norfenfluramine cause the release of serotonin from the rat ileum and, furthermore, it confirms the observation that agents that increase cytosolic calcium, such as caffeine and potassium chloride (KCl), also cause release of serotonin. As dexfenfluramine is known to inhibit voltage-dependent potassium (Kv) channels (23), we also examined the effect of the classic Kv channel blocker 4-aminopyridine (4-AP) on serotonin release. These observations on release could provide important insights into the mechanisms responsible for anorectic-induced pulmonary hypertension and possibly for PPH itself. Although dexfenfluramine is no longer marketed, an understanding of these mechanisms would provide a means of screening new anorectic agents. In addition, the results reported here may help explain the elevated levels of serotonin observed in PPH.

METHODS

Perfusion of rat ileum. Male Sprague-Dawley rats (250–350 g) were anesthetized with ketamine and xylazine and ventilated through

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a tracheostomy. After a median laparotomy, the most distal 20 cm of the ileum were isolated from the rest of the intestine. The superior mesenteric artery was perfused (1 ml/min) by a peristaltic pump (Masterlab, Microprocessor Pump Drive, Cole-Parmer Niles, IL) with Earle’s physiological solution (in mM): 116 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.4 MgSO₄, 1 NaH₂PO₄, 5.5 glucose, 26.2 NaHCO₃, and gassed with 95% O₂-5% CO₂, as previously described by Holzer and Lembeck (9). After an equilibration period (70 min), three control samples of effluent from the inferior mesenteric vein were drained into HPLC sample tubes for 1-min periods at 70, 80, and 90 min for measurement of serotonin. These served as a baseline. After drug administration (described in RESULTS), additional timed samples of the effluent perfusate were collected and stored at −20°C until the measurement of serotonin was performed. The pH, O₂, and CO₂ tensions of the perfusate were determined with a blood gas analyzer (Rapidlab 348, Bayer, Norwood, MA).

**Flow correction.** In the course of the experiments (70–90 min), the efflux of perfusate tended to decline (from 0.77 ± 0.03 to 0.69 ± 0.03 ml/min). Consequently, if the release of serotonin were to be constant and no allowance for the volume of perfusate was made, the calculated concentration would rise during the experiment. To avoid this potential error, the concentration in nanograms per milliliter of each sample was corrected for the decrease in perfusion volume by multiplying the measured concentration by the ratio of the venous flow rate (out) and the arterial flow rate (in).

**Serotonin determination by HPLC.** Serotonin in the stored samples was determined by HPLC (Beckman-Coulter System Gold) with fluorometric detection (Jasco FP-1520), after single-step deproteinization with perchloric acid (2, 13). Samples were thawed at room temperature and kept on ice. Fifty microliters of N-methyl-serotonin (NMS; 1 μg/ml) were added to the 250-μl sample, as an internal standard. Also, 7 μl of perchloric acid were added and the mixture was vigorously vortexed for 30 s. Samples were left on ice for 15 min for complete precipitation of proteins and then centrifuged at 12,000 g for 5 min at 4°C using an Eppendorf microcentrifuge. Serotonin was determined after injection of 25 μl of samples into a 3.9 × 300-mm reverse-phase Resolve C18 analytic column (Waters) packed with 5-μm beads. Stock solutions of serotonin and NMS (1.0 mg/ml) were prepared with HPLC grade water and stored in amber tubes at 4°C. The working standards were prepared fresh daily by diluting the stock solution to required concentrations. The mobile phase (0.1 M sodium acetate, 0.1 M citric acid, 1 mM dibutylamine, 0.5 mM sodium octyl sulfate, and 0.1 mM disodium EDTA containing 150 ml/l methanol) was prepared fresh daily with HPLC grade water, buffered with 0.05 M acetate, 0.1 M citric acid, 1 mM dibutylamine, 0.5 mM sodium octyl sulfate, and 0.1 mM disodium EDTA containing 150 ml/l methanol) was prepared fresh daily with HPLC grade water, filtered under a vacuum using a 0.2-μm filter, and degassed at room temperature before use. The mobile phase was delivered at a flow rate of 0.8 ml/min. Fluorometric detection was accomplished using excitation and emission wavelengths of 285 and 340 nm, respectively. For calculation of concentrations using peak areas with respect to internal standards, the Beckman software, Karat 32, was used. Data are expressed as nanograms per milliliter of sample.

**Statistics.** Values are expressed as means ± SE. Changes in serotonin values between the different drug groups and the control group were compared by a repeated-measures ANOVA. When the ANOVA indicated significance, the groups were compared using Fisher’s projected least-significant difference. A value of P < 0.05 was considered significant.

**RESULTS**

Studies on isolated intestinal preparations from a number of species indicate that serotonin is spontaneously secreted at a consistent baseline level and that pharmacological agents can increase or decrease this secretory activity (21, 26). After the equilibrium and control periods, the preparations were exposed to 20 min each of increasing concentrations (1, 10, and 100 μM) of dexfenfluramine (n = 6), D-norfenfluramine (n = 6), or the SERT inhibitor fluoxetine (n = 6), and samples of the perfusate were collected at regular intervals for serotonin content analysis. Reservoirs containing the different concentrations were bubbled and prewarmed to 37.8°C (physiological rat body temperature). In control studies (n = 8), the perfusate was switched from one reservoir to another at the corresponding intervals but no drug was added. The serotonin concentration in the perfusate (Fig. 1) was increased significantly after dexfenfluramine (100 μM) and D-norfenfluramine (10 and 100 μM). Fluoxetine did not have a significant effect on the serotonin release from the gut, suggesting that inhibition of reuptake alone was insufficient to increase serotonin levels in the perfusate. There was no difference in the wet weight of the ileum at the end of the experiments among the four groups.

In a separate experiment, a voltage-gated potassium channel blocker 4-AP (5 mM) was added to the perfusate (n = 3) after the 30-min baseline period and caused a sustained increase in serotonin release compared with the control period (Fig. 2). In the third experiment, KCl (60 mM, n = 5) caused a brief increase in corrected serotonin concentration (Fig. 3) that was
not statistically significant. KCl had a greater effect in reducing perfusate efflux, presumably because of vasoconstriction. The uncorrected serotonin concentration rose from 2.2 ± 0.3 ng/ml to a peak of 6.6 ± 1.0 ng/ml (P < 0.05). It is not apparent whether the principal loss of perfusate occurred before or after the vascular bed where serotonin is released. If the loss occurred before the bed, correction for volume is appropriate; if after, the serotonin concentration would not be affected and correction is not required.

Finally, caffeine, which releases calcium from the sarcoplasmic reticulum of most cell types, caused a sustained, significant increase in serotonin release from the ileum (30 mM, n = 4). Figure 4 illustrates the change in serotonin due to caffeine.

DISCUSSION

The observation by Herve et al. (8) that plasma serotonin levels are elevated in patients with PPH, even after bilateral lung transplantation, suggests that serotonin either plays an etiologic role or is a marker for an etiologic agent. As serotonin metabolism does not appear to be reduced (12), it is possible that the increased plasma serotonin levels reflect increased production by the EC cells, predominantly in the ileum. This study shows that dexfenfluramine and its major metabolite d-norfenfluramine increase serotonin release from the rat ileum. The observation raises the possibility that serotonin, or a substance released concomitantly, may be involved in the etiology of PPH occurring secondary to anorectic drugs. This possibility is strengthened by the finding that fluoxetine, a widely used serotonin-reuptake inhibitor that is not associated with PPH, did not increase serotonin release from the ileum. This suggests that the ability of dexfenfluramine and d-norfenfluramine to increase effluent serotonin concentration relates to increased release rather than inhibition of reuptake.

It has previously been reported that anorectic agents, such as dexfenfluramine and aminorex, can increase cytosolic calcium in PASMCs by two mechanisms. One is the inhibition of outward K+ current (I_{K}), leading to membrane depolarization and calcium influx through the voltage-dependent Ca^{2+} channels (22, 31). The other is through the release of calcium from the sarcoplasmic reticulum. It is important to note that fluoxetine does not cause inhibition of I_{K} at or near resting membrane potential and could not elevate cytosolic Ca^{2+} by this mechanism (22). The common feature of fluoxetine, dexfenfluramine, and d-norfenfluramine is the ability to block SERT. The fact that fluoxetine does not cause PH suggests that inhibition of SERT is not critical to the mechanism of anorexin-induced pulmonary hypertension.

Based on this prior experience, we examined the effect of a classic Kv blocker, 4-AP, on serotonin release. The increase in release is shown in Fig. 2. Similarly, switching to a high-K+
concentration in the perfusate, which would cause membrane depolarization, tended to cause serotonin release, as described by others (21, 27). High K+ and 4-AP would be expected to cause an influx of calcium and it has been reported that the inhibition of L-type calcium channels with nifedipine or the removal of external calcium (21) will markedly reduce serotonin secretion. Release of serotonin from nerve endings from the human cerebral cortex by low levels of dexfenfluramine is also calcium dependent (3). In isolated cell electrophysiology and calcium fluorescence experiments on PASMCs, the concentration of dexfenfluramine (100 μM) required to cause membrane depolarization is greater than that (10 μM) required to increase cytosolic calcium (22). This suggests that a lower concentration of dexfenfluramine may release calcium from the sarcoplasmic reticulum or cause calcium influx through non-voltage-dependent plasmalemmal channels. If this is also true for EC cells, it may explain the relatively brief release of serotonin in response to 60 mM KCl compared with the sustained release caused by caffeine.

The serotonin release into the perfusate in response to both dexfenfluramine and nortryptiline is maximal at ~10 μM and no further increase was seen at 100 μM. The plasma concentration of dexfenfluramine seen in patients on the drug was probably somewhat <1 μM (4). It is remarkable that the acute effects (a 50 to 300% increase in serotonin release) of these drugs occur in rats that are not genetically prone to have high plasma serotonin levels such as are seen in fawn-hooded rats and PPH patients. The effect of dexfenfluramine was apparent at concentrations 10 to 100 times the clinically observed plasma level. It is also possible that dexfenfluramine may be concentrated over time in the mesenteric and pulmonary interstitium of patients, compared with the plasma.

Elevated levels of serotonin have been reported in patients taking fenfluramines (17) as well as in rats given dexfenfluramine and exposed to mild hypoxia (6). On the other hand, fluoxetine has no effect on plasma serotonin (20) in rats in vivo, although it does increase serotonin in platelet-free plasma (20). Increased plasma levels of serotonin in the "serotonin hypothesis of anorectic-induced PPH" (28) have been thought to be the result of decreased uptake and/or increased release of serotonin by platelets. However, dexfenfluramine causes release of serotonin from human platelets only at concentrations 100 times higher than those detected in plasma during the treatment of obesity (10). Consequently, the elevated plasma levels of serotonin might be due, in part, to decreased uptake through the serotonin transporter. However, the lack of a link between fluoxetine and PPH, discussed above, suggests that decreased uptake alone is not sufficient to cause PPH. We conclude, therefore, that the increased release from the ileum may be a significant component of the elevated serotonin levels documented by Herve et al. (8) and could contribute as "a second insult" to the development of PPH, unrelated to anorectic agents. The observation that serotonin levels are elevated in PPH patients, even after bilateral lung transplantation, indicates that either serotonin or substances genetically linked or secreted with serotonin must be playing a part in the etiology of PPH. K+ current is known to be reduced in the PASMCs of PPH patients compared with those with secondary pulmonary hypertension (32). One can speculate that if K+ channel expression or function is also reduced in the EC cells of the PPH patients, this, as with dexfenfluramine, nortryptiline, and 4-AP, and KCl, might lead to increased secretion of serotonin and possibly other growth factors that contribute to the etiology of PPH.

GRANTS

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