Plasma Levels of 5-HT and 5-HIAA Increased after Intestinal Ischemia/Reperfusion in Rats

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Abstract: Intestinal ischemia/reperfusion (I/R) causes serious systemic injury, mainly from a variety of bioactive substances released from the injured intestine. To assess the possible roles of serotonin (5-hydroxytryptamine, 5-HT), a bioactive amine mainly stored in the intestine, in I/R injury, we assayed the levels of tryptophan, 5-HT, and 5-hydroxyindole acetic acid (5-HIAA) in the blood and intestine in a rat I/R model. Plasma 5-HT increased significantly over time after reperfusion; the plateau level was obtained 4 h after reperfusion and was associated with an increase in 5-HIAA. Plasma tryptophan levels declined gradually after reperfusion. The ratio of 5-HIAA/5-HT was significantly higher in I/R rats than in control rats, suggesting that elevated 5-HT was quickly metabolized in the systemic circulation. In the intestine, 5-HT decreased dramatically, whereas tryptophan increased. This phenomenon was prominent in the severely damaged intestine. These findings suggest that the injured intestine released large amounts of 5-HT, whereas its synthesis in the injured intestine was suppressed. An increase in 5-HT in the circulation may be related to various circulatory disturbances observed in humans after intestinal ischemia. [Japanese Journal of Physiology, 48, 333–339, 1998]

Key words: serotonin, 5-HT, intestine, ischemia/reperfusion, reperfusion injury.

Intestinal ischemia/reperfusion (I/R) induce severe systemic injury manifested by altered microvascular and epithelial permeability, hemorrhage, and villus necrosis. Severe lung, liver, and kidney damages with concomitant dysfunction of the systemic circulation are reported in patients with I/R and lead to a poor prognosis [1, 2]. Such systemic disorder after I/R is speculated to be induced by many kinds of bioactive substances that are released from the injured intestine after reperfusion. Endotoxin is known to be translocated from intestinal contents to the systemic circulation because of an increased permeability of the intestinal mucosa after ischemia [3]. Plasma levels of platelet-activating factor (PAF) [4–6], oxygen radicals [7, 8], histamine [9, 10], and TNF-α [11, 12] are also reported to increase after I/R, causing multiple organ failures. These substances are known to play important roles in the systemic injury after I/R directly or indirectly by increasing other mediators such as nitric oxide [13].

Serotonin (5-HT) is a bioactive amine that functions in a variety of physiological events such as neurotransmission, intestinal motion, platelet activation, and vasoconstriction mediated through at least 14 different kinds of receptors [14]. Under normal physiological conditions, most 5-HT in the whole blood is contained in platelets, and only a fraction is circulated in the plasma. The activation of platelets, therefore, is known to increase plasma 5-HT level and initiates several physiological events such as vasoconstriction or further activation of platelets. Pulmonary edema associated with pulmonary hypertension is also reported to be induced by elevated 5-HT in plasma [15].

Enterochromaffin cells in the small intestine also store 5-HT, where it is synthesized from tryptophan by tryptophan-hydroxylase. Considering the physio-
logical and pathological functions of 5-HT, we hypothesized that 5-HT released from the injured small intestine might play an important role in a variety of systemic disorders observed after I/R. In the present study, we assayed the levels of 5-HT and its derivatives in the plasma and in the injured intestine over time after I/R in a rat model. Results obtained in the present study suggest that plasma 5-HT significantly increased after I/R, probably because of an increased release of 5-HT from the injured intestine. Employing several different kinds of 5-HT agonists and antagonists, we also tried to assess the mechanisms and roles played by 5-HT in I/R.

METHODS

The studies were performed on male Wistar rats weighing 200–230 g. The experimental animals received food (standard laboratory chow) and tap water ad libitum. The animals were allowed at least 1 week to acclimatize to the environment before experiments were performed. They were anesthetized with sodium pentobarbital (40 mg/kg body wt; I.P.), which was supplemented thereafter as needed.

Experiment 1: Measurement of blood 5-HT levels after I/R. The extra jugular vein was exposed, and a polyethylene tube was inserted to obtain blood directly from the superior vena cava. After laparotomy performed through a midline abdominal incision, the small intestine was reflected to the left, and the superior mesenteric artery (SMA) was isolated. The animals were divided into two groups: an I/R group and a control group. In the I/R group (n=5), the small intestine was subjected to 1-h ischemia by applying a microvascular clip to the origin of the SMA and reperfusion followed by removing the clip. The laparotomy incision was closed immediately after reperfusion. Blood samples (0.7 ml) were collected through a polyethylene tube (coated with heparin) before the induction of ischemia and 0, 1, 2, 3, 4, and 5 h after reperfusion. In sham-operated control animals, the identical procedure was performed without the placement of the microvascular clip on the SMA (control group, n=5). Whole blood was mixed with 1/10 vol. of 0.11 M sodium citrate. To obtain platelet-poor plasma, the samples were centrifuged at 10,000×g for 5 min at 4°C. Whole blood or plasma (250 μl) was added with 50 μl of 1.14 M ascorbic acid and 50 μl of 3 M perchloric acid (pH 3.0) and was centrifuged at 30,000×g for 10 min at 4°C. The supernatant was used immediately or stored at −80°C until assayed.

Experiment 2: Measurement of intestinal 5-HT levels after I/R. The animals in this experiment were divided into two groups: an I/R group (n=7) and a control group (n=7). Identical procedures as in Experiment 1 were applied to these groups. Four hours after reperfusion, the intestines were removed and the three sections were resected from each intestine as samples. The upper section was a 5 cm segment of the jejunum, starting approximately 5 cm from the gastroduodenal junction. The middle section was a 5 cm segment of the ileum, starting 20 cm proximal to the ileo-cecal junction. The lower section was a 5 cm segment of the ileum, starting 10 cm proximal to the ileo-cecal junction. The intestines were rinsed by gentle flushing with ice-cold physiological saline. The samples were quickly frozen and stored at −80°C until the following procedures were performed. Frozen tissues were homogenized in 0.15 M perchloric acid with 670 μM EDTA (pH 3.0). The samples were then centrifuged at 30,000×g for 10 min at 4°C, and the supernatants were stored at −80°C until assayed.

Experiment 3: Analyses of the effects of several 5-HT agonists and antagonists on the survival rate 24 h after reperfusion. These animals were divided into six groups: In the 1st group (n=22), rats were subjected to 1-h ischemia as in Experiment 1. In the 2nd group (n=22), rats were injected with 5-methoxytryptamine hydrochloride (5MeOT) [14, 16–18] at a dose of 3 mg/kg I.P. 30 min before 1-h ischemia. In the 3rd group (n=10), rats were injected with 5MeOT at a dose of 3 mg/kg I.P. and were subjected to laparotomy alone without ischemia, which was considered as control for the 2nd group. In the 4th group (n=10), rats were injected with tandospirone [19–21] at a dose of 10 mg/kg I.P. 30 min before 1-h ischemia. In the 5th group (n=11), rats were injected with sarpogrelate [21–23] at a dose of 2 mg/kg I.P. 30 min before 1-h ischemia, and in the 6th group (n=11), rats were injected with SB204070 [16, 17, 24] at a dose of 2 mg/kg I.P. 30 min before 1-h ischemia. After the abdominal incision was closed, the animals were allowed to waken, and the survival rate was assessed 24 h after reperfusion.

Assays (measurement of 5-HT–related substances). Quantitative measurements of tryptophan, 5-HT, and 5-HIAA in whole blood and plasma were made by using the HPLC method described by Anderson et al. [25]. HPLC consists of a Beckman 110B pump (Beckman, USA), an Inertsil ODS-2 reversed-phase column (GL Sciences, Inc., Tokyo, Japan), an RF-535 fluorescence detector (Shimadzu, Kyoto, Japan), and a Chromatopac C-R6A data processor (Shimadzu). Excitation and emission wavelengths were set at 285 and 345 nm, respectively.
Plasma 5-HT after Intestinal Reperfusion

solvent system, a mixture of 950 ml of sodium acetate (pH 4.0, 10 mM) and 50 ml of methanol, was delivered at a flow rate of 2 ml/min. The 20 μl sample was injected into the chromatograph. The detection limits for tryptophan, 5-HT, and 5-HIAA were all 20 pg.

Quantitative measurements of tryptophan, 5-HT, and 5-HIAA in intestinal tissues were made by using HPLC with an electrochemical detector. The electrochemical apparatus consisted of a reverse phase HPLC column (Eicom Co., Kyoto, Japan). The column was a MA-50DS (2.6×150 mm) with a mobile phase composed of 0.1 M sodium acetate, 0.1 M citric acid, 15% methanol, 9 mM 1-octane sulfonate, and 13 μM EDTA. The mobile phase was delivered by a pump at a flow rate of 0.3 ml/min. The column temperature was kept at 25°C. The graphite electrode (WE-3G: Eicom Co.) was set at 0.8 V (an Ag/AgCl reference electrode). The detection limit for 5-HT was 1 pg.

Materials. 5MeOT and SB204070 ([1-butyl-4-piperidinyl-methyl]-8-amino-7-chloro-1,4-benzodioxan-5-carboxylate hydrochloride) were provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Sarpropreglate (±)-1-[o-[2-(m-methoxyphenyl)ethyl]-phenoxy]-3-(dimethylamino)-2-propyl hydrogen succinate hydrochloride was a gift from Tokyo Tanabe Co., Ltd. (Tokyo, Japan). Tandosiprone was a gift from Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). These reagents were dissolved in 0.9% w/v saline and administered to the rats at a volume of 2 ml/kg body weight. Rats receiving no drug treatment were given the same amount of 0.9% w/v saline.

Ethics. The study was submitted to the Ethical Committee of the Hamamatsu University School of Medicine. The committee judged the experimental designs according to “the guiding principles for the care and use of animals in the field of physiological sciences” recommended by the Physiological Society of Japan on December 19, 1988. These guidelines were prepared to meet the requirements contained in such publications as Guide for the Care and Use of Laboratory Animals, DH EW Publication No. (NIH) 85-23, 1985, and International Guiding Principles for Biomedical Research Involving Animals, CIOMS, 1984. The committee approved the experiments after careful examination.

Statistical analyses. Group differences were tested for significance by using repeated measures ANOVA in Experiment 1, two-factorial analysis for variance followed by post hoc analysis using Fisher’s PLSD in Experiment 2, and Fisher’s exact probability test for independence in Experiment 3. A p value of <0.05 was considered statistically significant. The results are expressed as mean±SEM.

RESULTS

Blood serotonin level after intestinal I/R

The effects of I/R on levels of whole blood and plasma 5-HT, tryptophan, and 5-HIAA are shown in Fig. 1. In the I/R group, whole blood 5-HT increased significantly from 2 h after reperfusion and reached a peak at 3 h after reperfusion (1.09±0.03 mg/ml), which was 1.3 times higher than in the control group (0.82±0.02 mg/ml) (Fig. 1A). Plasma 5-HT increased significantly from 1 h after reperfusion and reached a plateau at 4 h after reperfusion (201±21 ng/ml), a level that was more than 7 times higher than the control level (38±5 ng/ml) (Fig. 1B). I/R had no effect on whole blood and plasma tryptophan levels (Fig. 1. C and D). Whole blood 5-HIAA increased significantly from 1 h after reperfusion and reached a plateau 3 h after reperfusion (439±63 ng/ml); the level at 4 h was approximately 15 times higher than that of the control level (29±2 ng/ml) (Fig. 1E). Plasma 5-HIAA also increased significantly from 1 h after reperfusion and reached a plateau 3 h after reperfusion (2,135±279 ng/ml); the level at 3 h was approximately 14 times higher than the control level (149±49 ng/ml) (Fig. 1F).

Changes in 5-HIAA/5-HT ratio in whole blood.

The metabolic rate of 5-HT in whole blood, as indicated by 5-HIAA/5-HT ratio, increased significantly 1 h after reperfusion and reached a plateau at 4 h after reperfusion (0.421±0.059); the rate at 4 h was more than 15 times higher than the control rate (0.027±0.002) (Fig. 2).

The macroscopic intestinal findings

In the I/R rats, the parts of the intestines obtained 4 h after reperfusion were necrotic and their walls were edematous. Such pathological changes were greatest in the middle section, but less so in the lower section and slightest in the upper section of the resected intestines. Only slight ulcerations were observed in the intestines of control rats. We measured 5-HT, tryptophan, and 5-HIAA levels in each section of the resected intestine separately.

Changes in intestinal 5-HT levels

The 5-HT levels after I/R in the intestine are shown in Fig. 3A. Four hours after reperfusion, 5-HT levels in the section of all the I/R group were significantly lower than in the section of the control group. The middle section, which showed the strongest ischemic
Changes in intestinal concentrations of tryptophan

Tryptophan levels in the intestine are shown in Fig. 3B. Four hours after reperfusion, tryptophan levels in the I/R group were significantly higher than in the control group. The highest value was obtained at the middle section, followed by the lower and upper sections.

Changes in intestinal 5-HIAA levels

No significant differences in 5-HIAA levels were noted between the control and the I/R groups (Fig. 3C).

Effects of 5-HT receptor agonists and antagonists on 24-h survival rate after I/R

To assess the effects of elevated plasma 5-HT levels as a whole, we compared the 24-h survival rate after I/R as a marker of systemic disorders. The survival rate in the group pretreated with 5-HT$_{1,2,4}$ receptor agonist 5MeOT before I/R was significantly lower (26%) than in the group undergoing I/R without pretreatment.
Plasma 5-HT after Intestinal Reperfusion

Fig. 3. Comparison of 5-HT contents (A), tryptophan contents (B), and 5-HIAA contents (C), in three different sections with or without I/R. Data are shown by mean±SEM (n=7). *p<0.05; ***p<0.001 vs. controls.

(64%), although none of the rats pretreated with 5MeOT without I/R died. The specific agonist of 5-HT1A receptor agonist tandospirone, however, did not worsen, but it slightly improved the survival rate (80%), suggesting that the 5-HT2 or 5-HT4 receptor may be responsible for the lower survival rate associated with 5MeOT.

**DISCUSSION**

Plasma 5-HT and 5-HIAA levels increased dramatically after I/R, reaching a plateau at 4 (Fig. 1B) and 3 h (Fig. 1F), respectively. Plasma tryptophan levels did not change after I/R (Fig. 1D). Four hours after reperfusion, intestinal damage was still observed, and the amount of 5-HT in the injured intestines was significantly lowered compared with that in the control group (Fig. 3A). These results suggest that large amounts of 5-HT are released from the injured intestine into the systemic circulation after I/R.

The intestine contains large amounts of 5-HT, which are synthesized from tryptophan by tryptophan-hydroxylase, and pooled in enterochromaffin cells (ECs) [26, 27]. Tryptophan is supplied to ECs either from blood flow or from degraded proteins in the intestine. Even though large amounts of 5-HT are stored in the intestine, there is no report so far concerning alterations of 5-HT levels in the injured small intestine after I/R. The basal levels of 5-HT and tryptophan in the intestine that are reported here are similar to previously reported levels [28]. Four hours after reperfusion, 5-HT levels in the intestines decreased dramatically (Fig. 3A), and tryptophan levels increased (Fig. 3B). The latter were most prominent in the middle section where ischemic injury was strongest. These results suggest that I/R caused the release of 5-HT from storage in the injured intestine, although its synthesis from tryptophan was suppressed. Both the suppression of tryptophan-hydroxylase activity and the impaired oxygen supply during ischemia may be the apparent reasons for the suppression of 5-HT synthesis. The impaired uptake of 5-HT by ECs could be another reason for a dramatic decrease in 5-HT levels in the injured intestine [26]. Platelets are indeed another possible source for the increased plasma 5-HT levels after I/R, since platelet activation naturally occurs under the pathologic conditions of multiple organ failures. Whole blood 5-HT level, however, was also shown to increase after I/R (Fig. 1B), suggesting that elevated 5-HT in plasma originated in some other places. Under normal conditions, increased plasma 5-HT is known to be taken up rapidly by platelets and stored in the cells [29, 30]. The amount of 5-HT released from the injured intestine appears to be too much to be taken up by platelets, and the excess remains in the plasma. Besides other bioactive substances such as endotoxin, PAF, and TNF-α, 5-HT was also shown to be released from injured intestine after I/R.

It is known that 5-HT is involved in a variety of physiological and pathological events. So far, 14 different kinds of specific receptor types and subtypes for 5-HT have been identified [14]. Increased plasma 5-HT is known to initiate several pathological reactions such as platelet activation, vasoconstriction, or vasodilation together with enhanced vascular permeability mediated through the 5-HT2 receptor [31]. These reactions could be involved in the pathogenesis of intestinal I/R injury, which is deeply related to mul-
ultiple organ failure.

The treatment of I/R rats with 5-HT₄ receptor agonist (5MeOT), which also stimulates 5-HT₁ and 5-HT₂ receptors, worsened their 24-h postreperfusion survival rate at a dose that was not lethal for rats with no I/R. These results suggest that 5-HT worsens the pathological condition in I/R through the 5-HT₁, 5-HT₂, or 5-HT₄ receptor. A specific agonist of the 5-HT₁ receptor in turn, however, slightly improved the survival rate, suggesting that the 5-HT₁ receptor is not responsible for the effects of 5MeOT. To assess the possible involvement of the receptors of 5-HT₂ and 5-HT₄ in I/R, we used specific receptor antagonists of these two kinds of 5-HT receptors and analyzed their effects on survival at 24 h after reperfusion. Both antagonists improved the survival rate after I/R. Since plasma levels of 5-HT at 4 h after I/R were also elevated as in the control group, such improvement in the survival rate is likely induced by an effective blocking of 5-HT from binding to its specific receptors. We therefore suggest that 5-HT₂ and 5-HT₄ receptors may be responsible for initiating the pathological conditions after I/R.

The 5-HT₂ receptor is widely distributed in peripheral tissues. It initiates the contraction of smooth muscles of the vasculature, urinary tract, and gastrointestinal tract, as well as the increase in capillary permeability. Both platelet aggregation by 5-HT itself and the enhancement of platelet aggregation induced by other agonists such as ADP [32] and collagen are mediated by the 5-HT₂ receptor [33]. Vasoconstriction together with increased capillary permeability and enhanced platelet activation readily results in the impairment of systemic circulation and in lung edema, both of which are often seen in I/R [34]. These phenomena mediated by the 5-HT₂ receptor, therefore, could be involved in the pathogenesis of I/R.

The 5-HT₄ receptors are distributed in many organs including the brain, intestines, urinary bladder, heart, and adrenal glands [16]. In the intestines, the stimulation of the 5-HT₄ receptor has a pronounced effect on smooth muscle tone, mucosal electrolyte secretion, and the peristaltic reflex. It has been suggested that 5-HT₄ receptor stimulation after I/R induces water loss, which results in the hypovolemia that is usually observed after I/R [34]. Oxygen consumption enhancement in the injured intestine because of increased metabolism as a result of the stimulation of the 5-HT₄ receptor together with the 5-HT₂ receptor can be considered a responsible factor in worsening the ischemic change in the intestines.

Recently we indicated that stresses induced by electric foot shock or water immersion restraint resulted in circulatory disturbances in the stomach [35, 36]. These disturbances may also be caused in part by changes in plasma 5-HT levels.

In conclusion, increased 5-HT levels, not in the platelets but in plasma, appear to be involved in a variety of pathological changes, and these changes may be mediated through both 5-HT₂ and 5-HT₄ receptors in I/R injury. Together with other bioactive substances, increased plasma 5-HT levels could play an important role in the pathogenesis of intestinal I/R injury.

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