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The effect of tryptophan administration on ileum contractility and oxidant status in mice

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Summary. L-Tryptophan (TRP) is the precursor amino acid for the synthesis of serotonin (5-HT). 5-HT is effective both on the food intake and gastrointestinal system contractility. The aim of this study was to search the effects of systemic TRP treatment on 5-HT levels of ileum and searching the effect of ileal contractility and oxidant status. Swiss-albino mice were divided into two groups: 1. Control, 2. TRP-treated (100 mg/kg/24 h, i.p., for 7 days). Body weights were recorded at the beginning and at the end of experiments. Acetylcholine-induced contractile responses in the isolated ileum were recorded on polygraph. Ileal tissue malondialdehyde and glutathione levels determined by spectrophotometric and ileal tissue 5-HT levels were measured by immunohistochemical methods. TRP treatment decreased body weight and increased ileal contractile response. In the TRP-treated group, ileum malondialdehyde levels increased and glutathione levels decreased. Immunohistochemical detection showed that ileal 5-HT levels were increased by TRP treatment. There is a relationship between increased oxidative stress and increased contractility in the ileal tissue of the TRP-treated animals. These effects may be related to increased ileal 5-HT synthesis.

Keywords: Tryptophan – Serotonin – Ileal contractility – Oxidant status – Immunohistochemistry

Introduction

TRP is the first amino acid to be recognized and transported across the blood–brain barrier (Fernstrom and Wurtman, 1972). TRP is also the precursor of 5-HT and melatonin which function as a neurochemical substrate for a variety of normal behavioral and neuroendocrine functions. The conversion of TRP to 5-HT occurs in enterochromaffin cells (EC) of the gut, blood platelets and the central nervous system (Peters, 1991). 5-HT is involved in central regulation of feeding behavior. Increase of 5-HT in the brain tissue is caused by decreased food intake (Rotter et al., 1996). 5-HT is found in various peripheral regions and is particularly concentrated in the gastrointestinal tract (GIT) (Leibowits and Shor-Posner, 1986). GIT is one of the body's most abundant source of 5-HT. Previous studies have shown that 5-HT can be produced in and released from entero-endocrine cells of the gut wall (Denes et al., 2003). Tryptophan 5-hydroxylase (TPH), an initial and rate-limiting enzyme in the biosynthesis of 5-HT was mainly localized in the distal part of small intestine (Noguchi et al., 1973). In the intestine, TPH and 5-HT are present in EC in the mucosa and in neuronal cells in the submucosal and myenteric plexus (Ekwall et al., 1998). Acethylcholine (ACh) plays a central role in the enteric function acting at muscarinic and nicotinic receptors (Bennett, 2000; Sabeur, 1996). The release of 5-HT from entero-endocrine cells may be stimulated by muscarinic cholinergic receptors (Denes et al., 2003). 5-HT is involved in the regulation of gastrointestinal motility (Yamamato et al., 1999). It has been demonstrated that 5-HT increases the gastrointestinal contraction's amplitude mainly via a cholinergic pathway (Nakajima et al., 1997). Furthermore, it has been reported that 5-HT influences the transport of nutrients (Salvador et al., 2000).

The most important damaging effect of free radicals on tissues is lipid peroxidation. Oxygen-free radicals cause cellular injury by inducing lipid peroxidation which results in functional and structural cell interactions. Lipid peroxidation can be evaluated by the formation of malondialdehyde (MDA) (Grisotto et al., 2000). Reduced glutathione (GSH) is an endogenous antioxidant found in all

animal cells (Zhao et al., 2001). Previous reports indicate that tissue injury induced by various stimuli is coupled with GSH depletion (Sener et al., 2003). Kurup and Kurup's results show that increased levels of lipid peroxidation products and decreased levels of GSH were found together with increased TRP levels in the dominant hemispher of the brain (Kurup and Kurup, 2002). However, there is no direct evidence showing the effect of TRP administration on oxidant-antioxidant status of intestinal smooth muscle. Thus, this study was designed to investigate the effect of systemic TRP administration on 5-HT levels, the effect of TRP administration on contractile response induced by ACh and KCl and the effect of TRP on oxidant-antioxidant status in mice ileum.

Materials and methods

Animals

Prior approval for this experiment was obtained from the Animal Experimentation Ethics committee at Gazi University. In the experiments, 24 adult male Swiss-albino mice (obtained from Animal Care and Breeding Unit of the Gazi University Medical Faculty Ankara, Turkey) weighing 40 ± 4 g were used. They were housed in separate cages and maintained on a light-dark cycle of 14 h light and 10 h dark and fed with chow (Korkuteli-Turkey) ad libitum and daily mean consumption was 15 g/100 g body weight (BW). They were used in compliance with the European Community guidelines for the use of experimental animals. Animals were divided into two equal groups:

1. Control group (treated with physiological saline solution (0.9% NaCl) at a dose of 0.2 ml/day, intraperitoneal (i.p.) injection, for 7 days).
2. TRP-treated group (L-TRP, Merck-9770, 100 mg/kg/day in 0.2 ml of saline solution, i.p. injection, for 7 days) (Erikson and Walinder, 1998).

Animals body weights were recorded at the beginning and at the end of the 7 days of experimental period. At the end of the experiments mice were sacrificed in the 2nd hour of photophase by decapitation under ether anesthesia.

Preparation of tissues for concentration-response curves

After the mice were decapitated, midline laparotomy was performed. Distal 10 mm of terminal ileum was located and removed rapidly. Isolated terminal ileal segment was gently cleared from the surrounding fat, nerves and connective tissue. The tissue was rinsed in warm Tyrode solution to remove residual fecal material. Isolated mice ileum was suspended in 10 ml organ bath containing warmed (37 °C) and aerated (5% CO_2 in O_2) tyrode solution with 2 g initial tension. The tissues were allowed to equilibrate for 45 min until a steady-state baseline was obtained. Tyrode solution was prepared as follows (mM) NaCl 136.8; $CaCl_2$ 1.8; KCl 2.7; NaH_2PO_4 0.42; MgCl 1.05; $NaHCO_3$ 11.9; glucose 5.6 at pH 7.4. Concentration-dependent contractility responses to KCl (10–80 mM) and ACh (10^{-7}–10^{-4} M) were determined in each ileum and were recorded with Grass Model 7 Polygraph (Grass Instruments Co., Quincy, MA) by using force displacement transducer (Model FTO3, Grass Instruments). The contractile responses obtained by agonist were estimated either by the change of g-tension or by the% of maximum (Max) response of each preparation.

Biochemical assays for oxidative reactions

In the meantime distal, ileum samples were obtained and freezed immediately by liquid nitrogen, then kept in a −70 °C deepfreeze until analyses were performed. MDA as the last step of lipid peroxidation (Casini et al., 1986) and GSH as antioxidant were measured in the ileal part of the intestine by spectrophotometrical methods (Aykaç et al., 1985). Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Briefly, tissue samples were homogenized in ice–cold trichloroacetic acid (1 g tissue in 10 ml of 10% trichloroacetic acid) in a tissue homogenizer (Heideloph Diax 900, Germany). After centrifugation of the homogenate at $2250 \times g$ for 10 min (Hermle Z 323 K, Germany), 750 µl of the supernatant obtained was added to an equal volume of 0.67% (w/v) thiobarbituric acid (TBA) and heated at 100 °C for 15 min. The absorbance of the pigment formed was measured at 535 nm. Lipid peroxide levels are expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\,M^{-1} \cdot cm^{-1}$.

The GSH levels were determined by the modified Ellman method (Aykaç et al., 1985). Briefly, after centrifugation of homogenate at $2250 \times g$ for 10 min, 0.5 ml of the supernatant obtained was added to 2 ml of 0.3 M Na_2HPO_4 2 H_2O solution. Two tenth milliliter of a solution of dithiobisnitrobenzoate (0.4 mg/ml in 1% sodium citrate) was added to the mixture and absorbance at 412 nm was measured immediately after mixing. The GSH levels were calculated using an extinction coefficient of $13{,}600\,M^{-1}\,cm^{-1}$.

Immunohistological studies

Ten mice from each group were examined immunohistochemically and one specimen was taken from each mouse yielding ten specimen for each group.

Preparation of tissue samples

Pieces of distal ileum were transferred immediately to 10% formaline saline, dehydrated in graded alcohols and embedded in paraffin wax. 4 µm sections were cut with a microtome (Leica SM 2000, Germany) and mounted on polylysine-coated slides.

Antibodies and staining procedure

Slides were de-waxed. Following dehydration through a descending ethanol series, endogenous enzymes were blocked using 1.2% hydrogen peroxide. In order to increase the immunoreactivity of formalin-fixed parafin wax sections on slides were incubated in 0.1% protease (Protease XXV, Cat # 9006-002, Neomarkers, USA) for 10 min at 37 °C. After washing in phosphate buffer, slides were blocked using normal goat serum prior to the application of a 1:100 concentration of monoclonal mouse anti-serotonin primary antibody (Lot # 020-4, Cat # N1530, DAKO Corparation, USA). Two phosphate buffer rinses preceded secondary antibody application, biotinylated antimouse lg (Lot # 080-1, Cat # K0673, DAKO Corparation, USA), 1:300 for 30 min. Diluation of secondary antibody was conducted according to the data sheet. Immunostaining epitopes was identified using the avidin biotin complex (ABC) method and visualized with the use of DAB (diaminobenzidine tetra hydrocloride, Lot # 080-1, Cat # K0673, DAKO Corporation, USA). Control slides were prepared using the same method omitting primary antibody. Afterwards, the slides were counterstained with hematoxylin for 1 min, dehydrated in graded ethanol and mounted in a convensional medium (Mikroskopie Entellan # 740212765, Merck, Germany).

Statistical analysis

Data are expressed as means ± SD. Data of two groups were compared with an analysis of nonparametric Mann-Whitney U-test by using SPSS

for Windows 7.0 pack because of 2 groups and less than 20 animals in each group. Values of $p < 0.05$ were regarded as significant.

Results

Body weight and food consumption results

The effect of TRP treatment on the body weight of animals are shown in Table 1. There was no difference in the body weight of control group at the end of 7 days of the experiment but there was significant decrease in the body weight of TRP-treated ones (13.6%) ($p < 0.05$).

Ileal contractility results

Ileal contractilities induced with KCl did not change in the TRP-treated group when compared to the control group. Ileal contractile response to ACh at the concentration of 10^{-4} M as g-tension change was increased significantly by TRP treatment (10^{-4} M; 1.03 ± 0.4 for the control group, 1.75 ± 0.65 for the TRP-treated group). Ileal contractions induced by ACh are shown in Fig. 1. 80 mM KCl-induced contraction was accepted as maximum (Max) and ACh-induced contractions were estimated as % of the Max value. Contractile responses of ileum due to ACh with the % of Max value were found to be significantly increased in the TRP-treated group at the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M ($p < 0.05$, $p < 0.001$, $p < 0.0001$, respectively).

Table 1. The effects of tryptophan treatment on the body weight

Treatment	Body weight (g)	
	Start of experiment	End of experiment
Control group ($n = 12$)	39 ± 1.1	40.7 ± 1.6
Tryptophan-treated group ($n = 12$)	40 ± 1.2	35.7 ± 1.2*

Each value is the mean ± S.D. The mice were treated with TRP (100 mg/kg/day, i.p.) for 7 days. Control group received an isovolumetric amount of physiological saline in the same manner. Animals were weighed at the begining end at the end of experimental period. Control and TRP groups were allowed access to food. Difference statistically significant: * $p < 0.05$ by Mann-Whitney U-test

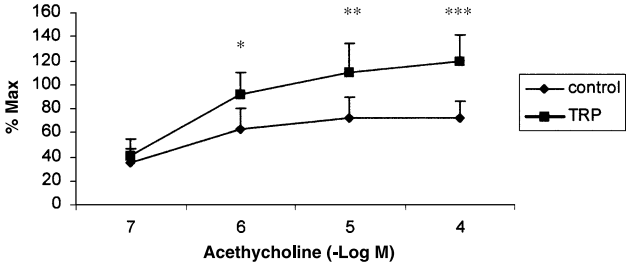

Fig. 1. % Maximum contraction to ACh in the isolated mice ileum. Each value is mean ± S.D. The mice were treated with TRP (100 mg/kg/daily, i.p.) in 0.2 ml saline solution for 7 days. Control group animals received an isovolumetric amount of serum physiological saline in the same manner. Concentration-dependent contractility responses to ACh (10^{-7}–10^{-4} M) were measured in each ileum and recorded with Grass Model 7 Polygraph. Difference statistically significant: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by Mann-Whitney U-test

Results of oxidative reactions

The MDA content of ileal tissue samples of control animals was 39.4 ± 8.7 nmol/g tissue, where as that of TRP treated animals was found to be 79.5 ± 10.7 nmol/g tissue (Table 2). TRP treatment resulted in approximately 2-fold increase in the MDA content of ileal tissue ($p < 0.0001$).

Administration of 100 mg/kg of TRP decreased the ileal GSH level significantly ($p < 0.001$). The amount of GSH measured in the ileal tissue sample of TRP treated animals was reduced approximately by 44% compared to that of control animals (Table 2). The relation between MDA level, GSH level and ileal contractions were not significant in the both groups. According to the Spearman's rho correlation coefficients were smaller than 0.4 in all relations.

Histological results

Control group

Weak to moderate membranous and very weak cytoplasmic 5-HT immunoreactivity was seen in absorptive cells. However, there was no staining in some absorptive cells. Strong and widespread reactivity was detected in some

Table 2. The effects of tryptophan treatment on ileum MDA and GSH levels

Treatment	MDA nmol/g tissue	GSH μmol/g tissue
Control group ($n = 12$)	39.4 ± 4.7	10.4 ± 1.8
Tryptophan-treated group ($n = 12$)	79.5 ± 6.7**	5.8 ± 0.9*

Each value is the mean ± S.D. The mice were treated with TRP (100 mg/kg/day, i.p.) in 0.2 ml of saline solution for 7 days. Control group animals received an isovolumetric amount of physiological saline in the same manner. Difference statistically significant: * $p < 0.001$, ** $p < 0.0001$ by Mann-Whitney U-test

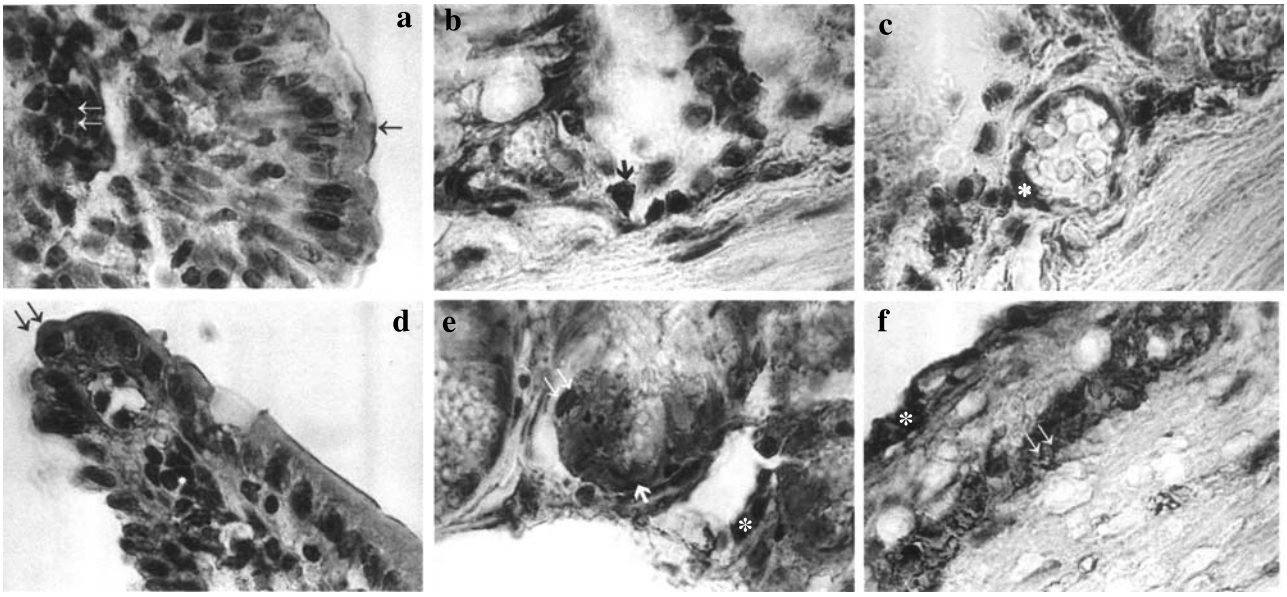

Fig. 2. 5-HT immunoreactivity of mice ileum tissues in the control and TRP treated groups. **a–c** 5-HT immunoreactivity of mice ileum tissues in the control group. **a** Weak to moderate membranous, and very weak cytoplasmic reactivity was detected in absorptive cells (↑). Strong reactivity was seen in some cells of connective tissue (↑↑) (Immunoperoxidase – hematoxylen ×100). **b** Moderate staining was observed in enteroendocrine cells in the basal part of intestinal gland (↑) (Immunoperoxidase – hematoxylen ×100). **c** Moderate to strong reactivity was seen in the blood vessel of tunica submucosa (∗) (Immunoperoxidase – hematoxylen ×100). **d–f** 5-HT immunoreactivity of mice ileum tissues in the TRP treated group. **d** Strong membranous and apical cytoplasmic staining was seen in absorptive cell (↑↑), moderate to strong reactivity was observed in villous connective tissue cells (∗) (Immunoperoxidase – hematoxylen ×100). **e** Moderate staining was observed in some cells of the basal part of intestinal gland (↑) and strong reactivity was seen in enteroendocrine cell (↑↑) and blood vessel of tunica submucosa (∗) (Immunoperoxidase – hematoxylen ×100). **f** Strong reactivity was seen in plexus myentericus (↑↑), and mesothelial cells of tunica seroza (∗) (Immunoperoxidase – hematoxylen ×100)

cells of villous connective tissue (Fig. 2a). In general, there were no staining basal parts of intestinal glands; however, moderate staining was seen in enteroendocrine cells (Fig. 2b). Moderate to strong 5-HT immunoreactivity was seen in the blood vessels of submucosa. No immunoreactivity was found in smooth muscle cells (Fig. 2c).

TRP group

Strong membranous and apical cytoplasmic 5-HT immunoreactivity was detected in absorptive cells. Moderate to strong reactivity was seen in some cells of villous connective tissue (Fig. 2d). Moderate staining was observed in some cells of basal parts of intestinal glands. Strong reactivity was seen in enteroendocrine cell and blood vessel of tunica submucosa (Fig. 2e). Strong 5-HT immnoreactivity was observed in plexus myentericus and mesothelial cells of serosa. There was no reactivity in the smooth muscle cells (Fig. 2f).

Discussion

The amino acid L-TRP is the precursor of the monoaminergic neurotransmitter 5-HT. It has been reported that i.p. administration of L-TRP to rats (100 mg/kg) resulted in an increase of TRP contents of plasma and brain (Erikson and Walinder, 1998). Another study showed that i.p. injection of TRP (150 mg/kg) to rats caused a highly significant increase of serum TRP levels (Huether et al., 1992). In our study we administered TRP (100 mg/kg daily) to rats by i.p. injection for 7 days. Although we did not directly measure the plasma TRP level in our experiments, we think that this dosage increased the plasma TRP level similarly as reported in other studies mentioned above. TRP is transported into the brain from the plasma through the blood–brain barrier (Erikson and Walinder, 1998). It has also been reported that when plasma TRP concentrations rise in rats receiving TRP, brain 5-HT concentrations also increase (Fenstrom and Wurtman, 1972). We observed by immunohistological detection that 7 days of i.p. TRP supplementation increased 5-HT levels of mice brain (Özer et al., 2002). Brain serotonergic mechanisms are important in the control of appetite. Injection of 5-HT to the paraventricular nucleus inhibits feeding, causing decreases in the size and duration of meals (Leibowitz and Shor-Posner, 1986). TRP administration also decreased body weight by 13.6% with the decreased daily food intake (Özer et al., 2004). These findings show that the increase in brain 5-HT

level leads to a decrease in body weight, and support the results of our study. In the present study, as compared with saline-injected controls, it was seen that the body weight of mice was decreased by i.p. administration of TRP for 7 days. In this study decreasing body weight in the TRP-treated group may be associated with the 5-HT increase in the brain and the effect of the increased 5-HT on the eating behaviour.

We also observed by immunohistochemical examination that i.p. TRP supplementation for 7 days increased 5-HT levels in the ileal part of the mice intestine. Gastrointestinal tract is the main source of peripheral 5-HT. Yu et al. (1999) demonstrated by the immunohistochemical method that tryptophan hydroxylase levels were high in the small intestine. They suggested that intestinal epithelium, EC, and mast cells in the gastrointestinal tracts may have the ability to synthesize 5-HT from TRP (Yu et al., 1999). In vivo microdialysis experiments indicating the activation of the 5-HT^4 receptor located in the gastrointestinal tract stimulate intestinal motor activity. Increased intestinal motor activity is associated with a local increase in ACh release from the intestinal cholinergic neurons (Taniyama et al., 2000). Salvador et al. (2000) reported that 5-HT increased the amplitude of contractions and integrated mechanical activity in the duodenum, jejunum and ileum of rabbit. Also, Salvador et al. (2000) suggested that 5-HT released from EC cells induced a local secretagogue effect and inhibition of the absorption of nutrient together with an augmentation of spontaneous and peristaltic activity (Salvador et al., 2000). Similarly, Nakajima et al. (1997) showed that i.v. 5-HT infusion increased regular cyclic patterns from the gastric antrum to the ileum of guinea pigs and also 5-HT increased the contractions' amplitudes at all sites. The authors observed that 5-HT induced contractions in the gastric antrum and duodenum were significantly inhibited by atropin and 5-HT antagonist and that in the ileum, only atropin inhibited 5-HT induced contractions. From these findings, it was suggested that 5-HT increased the gastrointestinal contractility amplitude mainly via a cholinergic pathway (Nakajima et al., 1997). In our experiments, ileal contractility was studied in the isolated organ bath. We used the KCl and ACh responses to classify changes in ileal contractility due to a nonreceptor mediated mechanism and a receptor mediated mechanism, respectively. Our results indicated that the KCl-induced nonreceptor-mediated contractile response did not change in the ileal smooth muscle of TRP-administered mice. But we found that i.p. TRP administration for 7 days significantly increased the contractile responses to ACh in the ileal smooth muscle.

There is no information on the relation between TRP, ileal contractility and oxidant stress. Therefore we determined MDA and GSH levels in the ileal tissue of mice given excessive TRP. Aviram et al. (1991) suggested that excessive dietary TRP increased plasma lipid peroxidation in the animals. LDL was peroxidized by incubation with copper ion in the presence of TRP or 5-HT. Thus 5-HT was to enhance in vitro LDL peroxidation. It has been implied that 5-HT or its metabolites enhance plasma lipid peroxidation via changes in the redox potential and in the lipid peroxidation chain reaction (Aviram et al., 1991). It has been reported that gastric mucosal thiobarbituric acid reactant levels are significantly increased after 2 days of 5-HT treatment (Yasuhiro et al., 1997).

In the present study, ileum tissue levels of MDA which are an important indicator of oxidative stress, were significantly increased. Ileum tissue levels of GSH which are an important antioxidant, were significantly decreased in TRP-treated mice as compared with control mice. Our result indicating the decrease in ileal GSH level of mice by TRP administration may be supported by the finding of Lee et al. (1998) that 5-HT stimulated elevation of superoxide anion generation in the pulmonary artery smooth muscle cells.

In this study, we demonstrated that systemic TRP administration for 7 days was effective in increasing 5-HT synthesis and ACh-induced contractile response of the ileum of mice. In addition to these findings, we observed an increase in oxidant stress and a decrease in antioxidant levels in the ileal tissue of mice given excessive TRP. Increased MDA levels were accompanied by increased 5-HT levels in the ileal tissue. Ileal contractile responses compatible with increased 5-HT levels were found in the TRP-administered mice.

In conclusion, these results indicate that there is a coincidence of increased oxidant stress, and an increase of contractility in the ileum of mice with excessive dietary TRP, and this suggests that both effects may be related to an increase in 5-HT level in the ileal tissue.

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