Nitroso-oxidative stress after activation of 5-HT\textsubscript{4} receptors under conditions of colitis in rats

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ABSTRACT
Serotonin (5-hydroxytryptamine, 5-HT) plays an important role in the regulation of the functioning of the gastrointestinal tract, including that of the colon. The response of smooth muscles, blood vessels and colon mucosa (CM) to 5-HT is realized through the activation of various types of 5-HT receptors, in particular, 5-HT\textsubscript{4} receptors, since the latter are identified on colon cells membranes (enterocytes, smooth muscles and endothelium). The aim of our study was to determine the effect of 5-HT\textsubscript{4} receptors agonist (mosapride) on nitrogen (II) oxide production and lipid peroxidation in CM and colon muscle tissue (CMT) under the conditions of experimental ulcerative colitis (UC).

ABBREVIATIONS AND SYMBOLS

INTRODUCTION
Development of UC is accompanied by oxidative processes due to the increased concentration of free radicals and the inadequate response of the antioxidant system. This results in different pathological changes in colon cells [1].

Inflammation in the CM and CMT brings about increased production of nitrogen (II) oxide (NO), a gaseous mediator, the main function of which is vasodilatation [2]. NO is produced by different isoforms of NO synthases. These are: inducible (activated by cytokines) and constitutive (endothelial and neuronal) [3]. Colon inflammation is accompanied by the activation of inducible NO-synthase (iNOS) that results in increased synthesis of peroxynitrite, a product of NO, and superoxide radical reaction, causing nitration and nitrosylation of cytoplasmic proteins [4]. Thereby, the increase of nitroso-oxidative processes is an important factor in the mechanism of UC development [5].

During inflammation, the activity of iNOS and cNOS changes [4] and this indirectly indicates changes in cytokines concentration. The effect of myeloperoxidase (MPO), reflecting the presence of neutrophils is similar, it produces hypochlorous acid and tyrosil radicals using hydrogen peroxide. Both of these are cytotoxic compounds and may aggravate lipid peroxidation [6].

The effect of 5-HT as a biologically active substance involved in regulation of colon function, is crucial [7]. The mucous membrane of digestive tract contains about 95% of the total 5-HT reserve of in the rat body. Of this, enterochromaffin cells (EC cells) synthetize 90% [8]. The distribution of EC cells in different parts of the digestive tract is equable – their content is approximately 0.25% when compared to other cells of digestive tract mucosa, but are higher (0.5%) in the area between the colon and rectum [9].

The complex interaction of bacteria, diet and intestinal cells affect synthesis, release and degradation of 5-HT, and therefore all the afore-mentioned factors may be responsible for the altered 5-HT function documented in many gastrointestinal diseases [10]. Indeed, abnormal regulation of 5-HT metabolism in the human gut was noted in numerous gastrointestinal disorders, in particular, in inflammatory bowel disease [11] and functional disorders such as irritable bowel syndrome [12]. Furthermore, alteration in 5-HT signaling was shown to be associated with celiac disease [13], colorectal cancer [14,15] and diverticular disease [16].

Release of 5-HT on neurons and smooth muscles of the CM affects secretion, reabsorption, microhemodynamics and motility [17]. The effect of 5-HT depends on the type of receptor that it binds to after being released from EC cells. Herein, the following types of 5-HT receptors have been identified on colon cells: 5-HT\textsubscript{1A, 1B, 1D}, 5-HT\textsubscript{2A, 2B, 2C}, 5-HT\textsubscript{3A}, 5-HT\textsubscript{4}, 5-HT\textsubscript{6} and 5-HT\textsubscript{7} [17].

Keywords: experimental colitis, mosapride, NO-synthase, lipid peroxidation, antioxidant enzymes.

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Nitroso-oxidative stress after activation of 5-HT4 receptors under conditions of colitis in rats

Nataliya Denysenko, Vitaliy Yemelyanenko, Iryna Fomenko, Alexander Sklyarov

Abstract

The activation of 5-HT4 receptors leads to release of acetylcholine from the effrent nerves of enteric nervous system, as well as 5-HT from the EC cells, causing colon muscle contractions. The effect of 5-HT on epithelial cells also involves 5-HT4 receptors, resulting in the CM increasing production of mucus, water and chlorine ions. The activation of 5-HT4 receptors leads to visceromotor sensitivity decrease. Thus, 5-HT signaling via 5-HT4 receptors is involved in regulation of intercellular integration of mucous and muscle tissues of the colon.

The aim of our study was to determine the effect of the 5-HT4 receptors agonist (mosapride) on the processes of cytoprotection in the CM and CMT in experimental UC, exploring the L-arginine/NOS/NO pathway, lipid peroxidation processes, antioxidant enzymes and MPO activity.

MATERIALS AND METHODS

The structure of this study and the experimental procedures performed on the animals were approved by the Ethical Committee of Lviv National Medical University (Protocol №3, 16.03.2015). The experiments were carried out in accordance with international guidelines for the use and care of laboratory animals.

Animals. 40 nonlinear white rats of both sexes, weighing 200-220 g were used. Rats were placed into 3 groups: 1 – intact animals, while UC was modeled in rats of groups 2 and 3. Animals of the 3 group were administered the 5-HT4 receptors agonist (mosapride) intragastrically and then UC was modeled. Drugs. 5-HT4 receptors agonist mosapride (“Mosyd MT™” tablets, Torrent Pharmaceuticals Ltd., India) was used in this research. Water suspension of tablets was prepared and mosapride was administered intragastrically via an orally introduced polyethylene tube at a single dose 10 mg/kg (volume of 1 mL) 1 hour before UC induction.

Procedure. UC was induced in animals of the 2nd and 3rd groups by introduction of 1 mL of 4% solution of acetic acid into the colon, which was pre-washed by saline. Exposure of acid solution was 1 min, then the colon was washed with 0.1 M phosphate buffer (pH = 7.4) [27]. Euthanasia was performed by intraperitoneal administration of urethane solution (Sigma, Switzerland) in a dose 3 g/kg.

After opening of the abdominal cavity, the colon was removed, cut lengthwise and washed with 0.9% sodium chloride, while the remaining moisture was removed with filter paper. Assessment of area and index of damage were performed by the planimetry method [28].

Lipid peroxidation level was determined due to malonic dialdehyde (MDA) concentration by measurement of the colored complex formed by MDA and thiobarbituric acid in acidic medium after boiling [29]. MPO activity was assessed through reaction of o-dianisidine oxidation by hydrogen peroxide, which is a substrate of MPO [30] and was expressed in IU/mg of protein. Superoxide dismutase (SOD) activity was measured by way of reaction of nitro blue tetrazolium reduction and formazan production and was expressed in IU·10⁶/mg of protein [31]. Catalase activity was determined due to peroxomolibdate (product of molybdate ammonium oxidation reaction) content and was expressed in μmol H₂O₂/min·mg of protein [32]. The activity of iNOS and cNOS was investigated through L-citrulline concentration measurement and was expressed in nmol of L-citrulline/min·mg of protein [33]. Arginase activity was assessed via urea concentration measurement and was expressed in μmol of carbamide/min·mg of protein [34]. Nitrites and nitrates concentrations were determined by reaction with Griess reagent and expressed in μmol/L [35]. Absorption was measured on biochemical analyzer StatFax 1904 (USA).

Statistical analysis. Statistical analysis was performed using OriginPro 7.0. Data are presented as the mean and standard deviation. The reliability was determined using the Student’s test because distribution, determined using the Shapiro-Wilk test, was normal.

RESULTS

Effect of acetic acid on CM (UC). Induction of UC led to emergence of deep erosions and ulcers in CM (Fig. 1.2) with the inflammation area 12.4±3.32 cm². The damage
Development of UC was accompanied by the increase of MDA concentration by 53% (p ≤ 0.001), while MPO activity increased 5.6-fold (p ≤ 0.001) compared to the control group (Tab. 1). In addition, the activity of antioxidant enzymes, SOD and catalase increased. Herein, activity of SOD rose 2.3-fold (p ≤ 0.001), activity of catalase increased 40% (p ≤ 0.001) compared to the control group (Tab. 1). The L-arginine/NOS/NO pathway under the conditions of UC was characterized by the following changes: iNOS activity increased 4.3-fold (p ≤ 0.001) and cNOS activity decreased 29% (p ≤ 0.001) compared with the control group of animals (Tab. 1). Intensive use of L-arginine by UC as a substrate led to reduced activity of arginase. This decreased 3.5-fold (p ≤ 0.001) compared to the control group. Furthermore, the concentration of L-arginine decreased from 94.71±23.71 μmol/L in the control group, to 68.8±11.36 μmol/L in the UC group (27%) compared to control (p ≤ 0.001). Nitrite and nitrate concentration also increased in the 2nd group of animals. Here, nitrite concentration rose 3.2-fold (p ≤ 0.001) and the total concentration of nitrite and nitrate increased 1.8-fold (p ≤ 0.001) compared to the control group (Tab. 1).

**Effect of acetic acid on CMT (UC).** Similar changes were observed in CMT: concentration of MDA increased 2.1-fold in the 2nd group (p ≤ 0.001) and MPO activity rose 4.8-fold (p ≤ 0.01) compared to the control group (Tab. 2). Another ratio of antioxidant enzyme activity was noted: SOD activity increased 60% (p ≤ 0.05) and catalase activity was enhanced by 87% (p ≤ 0.01), compared to the control group (Tab. 2). Moreover, activity of iNOS increased 3.4-fold (p ≤ 0.001) and cNOS activity decreased 17% (p ≥ 0.05) compared to control (Tab. 2). However, arginase activity did not change significantly (28%) (p ≤ 0.05) compared to control group (Tab. 2). A different concentration ratio was observed in CMT – nitrite concentration increased 45% (p ≤ 0.05) and total nitrite and nitrate rose 77% (p ≤ 0.001) compared to the control group (Tab. 2).

**Effect of mosapride on CM (in conditions of UC).** Administration of 5-HT₄ receptors agonist (mosapride) under conditions of UC led to decrease of inflammation area to 7.4±1.03 cm², or 60% (p ≤ 0.01) lower compared to the UC group (Fig. 1.3). After administration of 5-HT₄ receptors agonist (mosapride), the damage index decreased to 7.8±2.02 points, that is 69% (p ≤ 0.01) lower compared to the UC group. In addition, administration of 5-HT₄ receptors agonist (mosapride) under conditions of UC resulted in decrease of the concentration of MDA by 22% (p ≤ 0.01) and MPO activity by 37% (p ≤ 0.05) compared to the UC group (Tab. 1). Furthermore, activity of antioxidant enzymes decreased simultaneously: SOD by 28% (p ≤ 0.01) and catalase by 16% (p ≤ 0.01), compared to the UC group (Tab. 1). The action of the 5-HT₄ agonist under the conditions of UC decreased iNOS activity by 31% (p ≤ 0.05), while cNOS activity increased by 19% (p ≤ 0.05) and arginase activity rose 2.8-fold (p ≤ 0.001) compared to the UC group (Tab. 1). In addition, the concentration of L-arginine increased in the 3rd group by 16%, compared to UC group (p ≥ 0.05) to 79.9±12.18 μmol/L. Still, nitrite and nitrate concentration did not change significantly – nitrite concentration decreased by 20% and total nitrite and nitrate concentration decreased by 6%, compared with the UC group (Tab. 1).

**Effect of mosapride on CMT (in conditions of UC).** Changes in CMT were more significant: MDA concentration decreased by 36% (p ≤ 0.01) and MPO activity decreased by 42% (p ≤ 0.05) compared to the UC group (Tab. 2). Similar changes were observed in SOD activity: it decreased to 22% (p ≤ 0.05) and catalase activity diminished by 26% (p ≤ 0.01) compared to UC group (Tab. 2). Activity of iNOS decreased to 63% (p ≤ 0.001), cNOS activity increased by 10% (p < 0.001), whereas arginase activity increased by 14% (p ≤ 0.05) compared to the UC group (Tab. 2). What is more, nitrite concentration decreased by 10% and total nitrite and nitrate concentration decreased to 36% compared to the UC group (Tab. 2).

Thus, activation of 5-HT₄ receptors led to the reduction of nitroso-oxidative processes and reduced inflammation.

**DISCUSSION**

Our research shows that the effect of the 5-HT₄ receptors agonist (mosapride) led to

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**Table 1.** Changes of antioxidant enzymes, MPO, NOS, arginase activity, concentration of MDA, L-arginine in CM

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>UC</th>
<th>UC + Mosapride</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA, μmol/L</td>
<td>195±29.97</td>
<td>298±30.97**</td>
<td>232±23.42**</td>
</tr>
<tr>
<td>SOD, IU/102/mg of protein</td>
<td>7.05±1.365</td>
<td>15.91±3.958**</td>
<td>11.39±3.392**</td>
</tr>
<tr>
<td>Catalase, μmol H₂O₂/min·mg of protein</td>
<td>18.14±4.173</td>
<td>25.37±2.235***</td>
<td>21.31±3.233**</td>
</tr>
<tr>
<td>MPO, IU/mg of protein</td>
<td>4.75±1.721</td>
<td>26.5±2.644***</td>
<td>16.72±5.609*</td>
</tr>
<tr>
<td>INOS, nmol of L-citrulline/min·mg of protein</td>
<td>162±44.15</td>
<td>702±136**</td>
<td>486±75.28*</td>
</tr>
<tr>
<td>C-NOS, nmol of L-citrulline/min·mg of protein</td>
<td>677±108</td>
<td>482±3.155</td>
<td>392±139</td>
</tr>
<tr>
<td>Arginase, μmol of carbamide/min·mg of protein</td>
<td>0.179±0.0654</td>
<td>0.051±0.0136***</td>
<td>0.141±0.0313***</td>
</tr>
<tr>
<td>NO₂⁻, μmol/L</td>
<td>0.871±0.1461</td>
<td>2.817±0.773***</td>
<td>2.263±0.317</td>
</tr>
<tr>
<td>NO₃⁻ + NO₂⁻, μmol/L</td>
<td>3.018±0.4989</td>
<td>5.513±0.773***</td>
<td>5.171±1.3387</td>
</tr>
</tbody>
</table>

Remark: p < 0.05 (**), 0.01 (***), 0.001 (****) compared with vehicle group; p < 0.05 (**), 0.01 (***), 0.001 (****) compared with UC

**Table 2.** Changes of antioxidant enzymes, MPO, NOS activity, concentration of MDA in CMT

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>UC</th>
<th>UC + Mosapride</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA, μmol/L</td>
<td>133.3±17.44</td>
<td>269±36.1***</td>
<td>208±22.8**</td>
</tr>
<tr>
<td>SOD, IU/10/mg of protein</td>
<td>21.22±2.92</td>
<td>33.5±7.46*</td>
<td>26.07±6.9</td>
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<tr>
<td>Catalase, μmol H₂O₂/min·mg of protein</td>
<td>17.2±2.26</td>
<td>32.24±7.42**</td>
<td>23.7±6.05*</td>
</tr>
<tr>
<td>MPO, IU/mg of protein</td>
<td>15.5±1.7</td>
<td>74.2±6.6*</td>
<td>42.8±18.9</td>
</tr>
<tr>
<td>INOS, nmol of L-citrulline/min·mg of protein</td>
<td>197±32.27</td>
<td>678.3±111.7***</td>
<td>248.5±67.42***</td>
</tr>
<tr>
<td>C-NOS, nmol of L-citrulline/min·mg of protein</td>
<td>475.6±45.7</td>
<td>395±90.7</td>
<td>436.2±79.21***</td>
</tr>
<tr>
<td>Arginase, μmol of carbamide/min·mg of protein</td>
<td>0.29±0.04</td>
<td>0.21±0.03*</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>NO₂⁻, μmol/L</td>
<td>0.353±0.0376</td>
<td>0.51±0.0254*</td>
<td>0.458±0.0502*</td>
</tr>
<tr>
<td>NO₃⁻ + NO₂⁻, μmol/L</td>
<td>2.65±0.66</td>
<td>4.68±1.84***</td>
<td>2.99±0.58*</td>
</tr>
</tbody>
</table>

Remark: p < 0.05 (**), 0.01 (***), 0.001 (****) compared with vehicle group; p < 0.05 (**), 0.01 (***), 0.001 (****) compared with UC
the reduction of destructive damage area and decrease of nitroso-oxidative stress under conditions of experimental UC. Herein, the activity of iNOS decreased after activation of 5-HT4 receptors, that, respectively, led to the decrease of peroxynitrite production. These changes are quite obvious, as the increase of iNOS activity is mediated by the high content of inflammatory cytokines in the area of inflammation [36].

UC is characterized by destructive changes in CM, accompanied by increased content of MDA, protein and mRNA expressions of TNF-α [37], NF-κBp65 [38], IL-1β, IL-17 [39], IL-2, IL-4, IL-5 [40], increased activity of SOD and MPO [41], increased concentrations of prostaglandin E2, and leukotriene B4, decreased total glutathione content [42], increased iNOS and COX-2 expression [43], as well as decreased activity of arginase due to bacterial lipopolysaccharides and flagellin admission, in addition to increased oxidative processes in the CM [44]. What is more, increased activity of iNOS under conditions of UC causes reduction of L-arginine concentration and arginase activity [45], indicating the redistribution of L-arginine utilization by NOS and arginase under conditions of inflammation.

Unidirectional changes of nitroso-oxidative processes were also observed both in CM and CMT under conditions of UC. Here, increased lipid peroxidation processes and toxic products of NO cause the development of destructive lesions in the CM and CMT dysmotility.

Acute increase of iNOS activity in smooth muscle locally in the areas of inflammation brings about dilatation and reduced motility [46]. This is facilitated by the presence of destructive lesions in the CM, accompanied by the growth of toxic substance permeability from the gut cavity into tissues and blood flow.

Another marker of inflammation is MPO activity, in our experiment, this increased 5.6-fold in CM and 4.8-fold in CMT under conditions of UC, compared to the control group. This was due to the activation of tissue neutrophils.

The substance iNOS, has a significant role in the development of UC. In our work, its activity grew 4.3-fold times in the CM and 3.4-fold in CMT and the effect was accompanied by nitrite anion production and the total number of nitrates and nitrates was affected.

Development of UC is accompanied by increased levels of 5-HT biosynthesis in EC cells in the CM [10]. Here, 5-HT released from EC cells affects motility and secretion, shows neuroprotective effect and regulates cell growth and the protective mechanisms that are involved in the inflammatory process through a paracrine mechanism of action [47]. Of note, in literature, an increased number of EC cells in the CM under conditions of UC was reported [48].

Despite the increase in the allocation of 5-HT from EC cells under UC conditions, colon motor function decreases in the area of inflammation due to smooth muscle dilatation induced by overproduction of NO [36].

The 5-HT4 receptors play a leading role in the regulation of motility, secretion and nociception of colon. They are located on smooth muscles, EC cells and on neurons of the enteric nervous system. Activation of the myenteral neuron 5-HT4 receptors brings about an increase of acetylcholine release and the activation of the motility, while the effect of 5-HT4 agonists on EC cell receptors induces the excretion of 5-HT in the extracellular space [49].

The anti-inflammatory, antioxidant and cytoprotective action of 5-HT4 receptors agonist mosapride involves several mechanisms. Among these are the direct influence of mosapride on cells, the reduction of cytokines production in the CM and the release of local biologically active substances (prostaglandin E2, histamine, vasoactive intestinal polypeptide) [50]. Increased proliferative processes due to increased content of growth factors can be one of the possible mechanisms of the cytoprotective effect of 5-HT4 receptors agonist. Increased motility may also be due to reduced bacterial lipopolysaccharide and flagellin admission. The factors that increase cytoprotection may also include enhanced mucus production by epithelial cells on the membrane on which 5-HT4 receptors are located [7]. Hence, the activation of 5-HT4 receptors under conditions of acetic acid induced UC, showed cytoprotective, antioxidant and anti-inflammatory effects and reduced nitroso-oxidative processes due to the involvement of different mechanisms of action.

CONCLUSIONS

1. UC manifested with destructive changes in CM, accompanied by the acute increase of MDA concentration and, consequently, lipid peroxidation processes, MPO, iNOS activity, while the activity of arginase decrease in CM and CMT indicates the dominant role of nitroso-oxidative processes in the development of structural damage in the CM in UC.

2. Intragastric administration of 5-HT4 receptors agonist (mosapride citrate, 10 mg/kg) reduced the severity of destructive lesions of CM, and minimized MDA concentration, MPO, iNOS activity, as well as the activity of antioxidant enzymes – SOD and catalase, indicating the reduction of nitroso-oxidative processes in colon, compared to the UC group.

3. Application of 5-HT4 receptors agonist mosapride citrate had cytoprotective, antioxidant and anti-inflammatory effects and can diminish nitroso-oxidative stress under conditions of acetic acid induced colitis in rat CM and CMT.

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