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Nitric oxide as an active substance in the enteric neurons of the porcine digestive tract in physiological conditions and under intoxication with bisphenol A (BPA)

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ABSTRACT

Bisphenol A (BPA) is an organic substance, which is commonly used in the production of plastic. It is known that BPA has the negative impact on the living organism, affecting among others the reproductive organs, nervous, endocrine and immune systems. Nevertheless the knowledge about the influence of BPA on the enteric nervous system (ENS) is extremely scanty. On the other hand, nitric oxide is considered to be one of the most important neuronal factors in the ENS. The aim of the study was to investigate the influence of low and high doses of BPA on neuronal isoform nitric oxide synthase – like immunoreactive (nNOS-LI) nervous structures in the various parts of the porcine gastrointestinal (GI) tract using double immunofluorescence technique. The obtained results show that BPA affects nNOS-LI enteric neurons and nerve fibers, and the character and severity of observed changes depend on the fragment of the gastrointestinal tract, part of the ENS and dose of the toxin. It should be pointed out that even relatively low doses of BPA (0.05 mg/kg body weight/day) are not neutral for the organism and may change the number of nitrergic nervous structures in the stomach and intestine. Observed changes are probably connected with neurotoxic activity of BPA, but the exact mechanisms of them still remain unclear.

1. Introduction

The innervation of the GI tract consists of two parts: the enteric nervous system (ENS) and extrinsic sympathetic, parasympathetic and sensory innervation [1,2]. The ENS is located in the wall of the esophagus, stomach and intestine. It is built of millions of neuronal cells grouped in the intramural ganglionated plexuses. The types and number of these plexuses depend on the animal species studied. In small mammals (for example rodents) the ENS consists of two plexuses: myenteric plexus (MP) located between longitudinal and circular muscle layers and submucous plexus – near the lamina propria of the mucosa [3]. In the small and large intestine of big mammals (such as the domestic pig) submucous plexus is additionally divided into two separate plexuses: outer submucous plexus (OSP) – right next to the internal side of the circular muscle layer and inner submucous plexus (ISP) – near the lamina propria of the mucosal layer [4,5]. The ENS takes part in the regulation of the majority of the GI tract activities, such as the intestinal motility, secretion of the digestive enzymes, absorption of the nutrients, intestinal blood flow and many more [6–10]. Enteric neurons are very diverse in their morphology, functions and neurochemistry [7,11,12]. The latter characteristic seems to be the most important indicator of the enteric neurons differentiation. So far, apart from acetylcholine – the main neurotransmitter of the ENS, several dozen of other active substances have been described in the nervous structures within the GI tract [5,10,13,14]. One of them is nitric oxide (NO).

Nitric oxide is a gaseous neurotransmitter and/or neuromodulator, which is synthetized from L-arginine with the participation of nitric oxide synthases [15,16], among which neuronal isoform of nitric oxide synthase (nNOS) shall be considered as the marker of nitrergic neurons [17,18]. Until now, the presence of nNOS has been described in the ENS of numerous species, including human, and the number of nitrergic neurons clearly depends on the mammals species and the fragments of the GI tract studied [19–23]. Nitric oxide in the ENS seems to be one of the most important inhibitory factors, which affects various functions of the digestive tract. Previous studies have shown NO – induced relaxatory effects on the gastrointestinal smooth muscles and inhibition of the secretion of electrolytes and intestinal hormones [24]. Moreover, it is know that nitric oxide is an important vasodilator, so that it may regulate mesenteric and intestinal blood flow [21,25].

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Nevertheless, many aspects connected with functions of NO in the enteric neurons are not clear. One of them is participation of nitrergic enteric neuronal cells in pathological processes within the intestine. Some previous studies have suggested the neuroprotective functions of NO within the ENS, but the knowledge of this subject is rather scanty [18,22,26]. On the other hand, it is known that enteric neurons are subjected to changes under the influence various intestinal and extra-intestinal disease processes and toxic substances in the food [1,2,4,8,27].

The aim of the present study was to determine the influence of bisphenol A (BPA) on the nitrergic neurons in the ENS of the porcine digestive tract. It should be pointed out that the selection of both toxic substance and animal species was not accidental. BPA is an organic substance commonly used in the production of the plastic [28]. It is presented in bottles, food containers, household goods, toys, paints, varnishes and many other everyday things. BPA shows multidirectional negative impact on the living organisms. It is known that this substance, due to its similarities to estrogen, acts on estrogen receptors, resulting in reproductive and metabolic disorders [28–30]. Moreover, BPA shows neurotoxic effects, manifested by deviations in dendritic and synaptic development, changes in the synaptic structures and abnormal spontaneous behavior [31–33]. These facts strongly suggest that BPA may also influence the enteric nervous system, which is one of the first barriers against toxic substances in the food. Admittedly, it is known that BPA can act on the intestinal barrier function and participates in the inflammatory processes within the GI tract [34,35], the knowledge concerning the influence of described substance on the ENS is rather scanty. In turn, as regards the selection of animal species, the domestic pig is considered to be a good animal model of the pathological processes occurring in the human organism due to neurochemical, physiological and biochemical similarities between these two species, which especially concern the ENS [4,14]. Therefore the results obtained during the present study may reflect the influence of BPA on the human enteric neurons.

2. Materials and methods

Fifteen immature female pigs of Piétrain x Duroc breed (approx. 8 weeks, about 20 kg body weight) were used during the present study. All animals were kept in the same typical laboratory conditions in boxes without any plastic elements. Pigs were fed with the complete feeding stuff in the quantity recommended by the producer. All experimental procedures were made up in line with the recommendations of the Local Ethical Committee of Experiments on Animals in Olsztyn (Poland) (decision number 17/2013). After adaptive period pigs were randomly divided into three groups of five animals. The first of them was the control group (C group). Pigs of this group received empty capsules. The second group (low dose group – LD group) consisted of animals treated with capsules including bisphenol A (Bisphenol A 50 g, Sigma Aldrich, USA, catalogue no 239658) in dose 0.05 mg/kg body weight/day. This dose for a long time was legally appointed as maximal permitted dose by European Food Safety Authority [36]. Due to some informations about the influence of such doses on the immune system EFSA temporarily reduced tolerable daily intake of BPA to 4 μg/kg b.w./day [37]. Final determination of TDI for bisphenol A was conditionally related to results of the further studies, and the present study fits in these aspects. The third group of animals (high dose group – HD group) was treated with BPA in dose ten times higher than mentioned above (0.5 mg/kg body weight/day). Capsules for all animals were administered in the same manner - once a day, before the morning foraging. Before the administration of BPA and once a week during the study animals were weighed to exact dosage adjustment of BPA.

After 28 days of BPA administration all animals were euthanized. First, the animals were premedicated with Stresnil (Janssen, Belgium, 75 μl/kg of body weight given intramuscularly) and after about 20 min intravenously administration of an overdose of sodium thiopental (Thiopental, Sandoz, Kundl-Rakúsko, Austria) was performed. Immediately after euthanasia the fragments (about 3 cm in length) of the particular parts of the digestive tract were collected. Fragments were collected from the same parts of the GI tract from all animals in the following order: stomach (the fragment of the fundus 15 cm before the pylorus), duodenum (10 cm after the pylorus), jejunum (70 cm after the pylorus), ileum (10 cm before the ileocaecal valve) and descending colon (from the place, where nerves from the inferior mesenteric ganglia supply the intestine). The fragments of the GI tract were prepared to immunofluorescence technique according to method described by Gonkowski et al. [38]. They were fixed in a solution of 4% buffered paraformaldehyde (pH 7.4, 1 h, room temperature – rt) just after the collection. Then tissues were rinsed in phosphate buffer (0.1 M, pH 7.4, at 4 °C) for three days and placed in 18% phosphate-buffered sucrose (at 4 °C) for at least three weeks. After this period the fragments of the GI tract were frozen at −22 °C, cut perpendicular to the lumen of the GI tract into 14 μm-thick sections with the cryostat (HM 525, Microm International, Germany) and mounted on the microscopic slides.

Sections of the GI tract were subjected to the typical double immunofluorescence method according to previous descriptions [39]. In short, this method consisted of the following stages: a) drying of slices of the GI tract (45 min, rt), b) incubation with “blocking solution” containing 10% normal goat serum, 0.1% bovine serum albumin, 0.01% NaN3, 0.25% Triton X-100 and 0.05% thimerosal in PBS (1 h, rt), c) incubation with the mixture of two antibodies raised in different species directed towards protein gene-product 9.5 (PGP 9.5, Biogenesis, UK, catalogue no 7863–2004, working dilution 1:1000; used here as a pan-neuronal marker) and nNOS (MercMillipore, DEU, catalogue no AB5380, working dilution 1:6000) (overnight; rt, in the humid chamber), d) visualization of complex of primary antisera bounded to appropriate antigen by the incubation with the mixture of two secondary antibodies conjugated with Alexa Fluor: Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 546 donkey anti-rabbit IgG (both antibodies from Invitrogen, Carlsbad, CA, USA, working dilution 1:1000) (1 h, rt.).

The routine control tests of the antibodies were performed to exclude non-specific labelling. These tests included pre-absorption of the antiserum with the appropriate antigen, omission and replacement of primary antiserum by non-immune serum.

The labelled fragments of the GI tract were observed under an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets. The evaluation of the percentage of nNOS-LI neurons and number of nitrergic fibers was performed according to the method previously described by Gonkowski [5]. The determination of the percentage of nNOS – like immunoreactive (nNOS-LI) neurons in each type of the enteric ganglia was made by the evaluation of at least 500 PGP-9.5 - positive neuronal cells (only neurons with clearly visible nucleus) for the presence of nNOS. In this study the number of neurons immunoreactive to PGP 9.5 was considered as 100%. In turn, the number of nNOS – positive nerves in the muscular and mucosal layers was identified by the counting of all nerves immunoreactive to nNOS in the microscopic observation field (0.1 mm^2). The counting of nerves was performed in 4 sections per animal (in 5 fields per section). Data concerning the number of cells and fibers were pooled and presented as a mean ± SEM.

The density of intraganglionic nerve fibers immunoreactive to nNOS was identified with arbitrary scale, in which (−) indicated the absence of nerve fibers, (+) - single fibers, (++) - rare fibers, (+++) – dense network of fibers, and (++++) - a very dense meshwork of nNOS-LI nerves.

To prevent double counting of the same nervous structures, the fragments of the GI tract included into the evaluation were located at least 200 μm apart from each other.

Statistical analysis was performed with an one-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison post hoc test using Statistica 12 software (StatSoft Inc., Tulsa, OK, USA). The