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Neuregulin 1 (NRG-1) as a neuronal active substance in the porcine intrahepatic nerve fibers in physiological conditions and under the influence of bisphenol A (BPA)

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Abbreviated title: Neuregulin-1 in the porcine intrahepatic nerve fibers.

Abstract

Bisphenol A (BPA) is a substance commonly used in the production of plastics. Previous studies have described that it shows multidirectional harmful effects on the living organism. It is known that BPA causes liver damage, but knowledge about the roles of intrahepatic nerves in these mechanisms is extremely scanty. On the other hand, the exact roles of some neuronal substances in the nervous structures located in the liver still remain unknown. One of such substances, which is allocated a role in the stimulation of cell survival is neuregulin 1 (NRG-1). The aim of the present studies was to investigate the distribution of NRG-1 -like
immunoreactive (NRG-1-LI) nerves in the liver in physiological conditions and under the influence of various doses of BPA using routine double immunofluorescence staining. The results (for the first time) show the presence of NRG-1 in the intrahepatic nerves, and co-localization of NGR-1 with neuronal isoform of nitric oxide synthase (nNOS) and vasoactive intestinal polypeptide (VIP). Moreover, it has been observed that high doses of BPA increases the density of NRG-1-LI intrahepatic nerves and the degree of co-localization of NRG-1 with VIP. These observations suggest that NRG-1 located in intrahepatic nerves may play functions in processes connected with liver damage and/or regeneration under the impact of BPA.

**Key words: bisphenol A, liver, neuregulin, VIP, nNOS, porcine**

Neuregulin 1 (NRG-1) is one of the most obscure neuronal substances, whose functions are not clear. This 44-kD glycoprotein, discovered in 1992, belongs to a group of neuregulins - epidermal growth factors family proteins (Holmes et al., 1992). NRG-1 may affect the epidermal growth factor receptor family and, consequently, increases cell survivability, as well as takes part in the apoptosis inhibition and angiogenesis (Ikeda et al., 2009). Previous studies have described the presence of NRG-1 mainly in the central nervous system, where it is involved in processes connected with higher neural activity, development of cerebral cortex and differentiation of oligodendrocytes (Law et al., 2004; Taylor et al., 2012; Kataria et al., 2018).
Contrary to the central nervous system, knowledge about the distribution and functions of NRG-1 within the peripheral nervous system is more fragmentary. This substance was found in dorsal root ganglia, nervous structures supplying the uterus and nerves in the gastrointestinal tract (Yue et al., 2013; Rytel 2018; Barberán et al., 2016). Although NRG-1 has been reported in hepatocytes, where it influences liver development (Viechover et al., 2001), to date there have been no studies concerning NRG-1 in the intrahepatic nerve fibers.

It is assumed that NRG-1 in the peripheral nervous system may affect the receptors of neuromediators and influence ion channels (Kataria et al., 2019; Salvany et al., 2019). NRG-1 also participates in adaptive and protective reactions by activation of Schwann cell regeneration during pathological processes and under the impact of harmful substances (Viehover et al., 2001).

One of such harmful chemical factors which affects NRG-1–positive nerves is bisphenol A (BPA) (Rytel, 2018). BPA is a synthetic substance, which is commonly used in the production of plastics. It is present in a wide range of everyday products, including food containers, bottles, toys, furniture, thermal papers, household equipment, paints and many others (Mikolajewska et al., 2015). BPA may activate estrogen receptors and shows various negative effects on the internal organs and systems including, among others, the nervous system, gastrointestinal tract, endocrine glands and reproductive organs (Rochester, 2013). Moreover, the relationships between exposure to BPA and the risk of various civilization diseases (such as hypertension, heart attack, stroke, cancer and diabetes) have been observed (Melzer et al., 2010, 2012; Rochester, 2013; Metz, 2016). Due to its multidirectional harmful effects, BPA has been banned in several countries, including Japan, Korea, Canada and some states of the USA (Chen et al., 2016; Metz, 2016). In 2011, The European Union also introduced a ban on the use of BPA in products for infants (Metz, 2016).
The liver, as the main organ responsible for the accumulation, decomposition and metabolism of chemical substances, is also exposed to the negative effects of BPA, as evidenced in the increase of liver enzyme levels in serum under the impact of this substance (Melzer et al., 2010). It is known that BPA, first of all, increases the risk of non-alcoholic fatty liver disease (Peyre et al., 2014), which is connected with the ability of this substance to intensify fatty acid synthesis (Peyre et al., 2014; Zhang et al., 2015) and stimulate triacylglycerol accumulation in the hepatic cells (Marmugi et al., 2012). Moreover, even very low concentration of BPA may cause DNA damage and induce the proliferation of the hepatic cells in vitro (Kim et al., 2018), which may suggest the hepatocarcinogenicity of this substance. Other studies have described BPA-induced liver injury associated with an increase in nucleus aggregation, necrosis and inflammatory cell infiltration in hepatocytes (Elswefy et al., 2016). However, it should be underlined that knowledge concerning the influence of BPA on liver innervation is extremely scarce (Thoene et al., 2018).

For this reason, the aim of the present study was to investigate the influence of intrahepatic nerves immunoreactive to NRG-1 and co-localization of this substance with vasoactive intestinal polypeptide (VIP) and neuronal isoform of nitric oxide (nNOS – used as a marker of nitrergic nerves), because all of these substances are known as factors taking part in protective and adaptive processes (Dejda et al., 2005; Cinelli et al., 2019). Moreover, it is known that both VIP and nNOS are involved in processes connected with the impact of BPA on the autonomic nervous system (Szymanska et al., 2018a, b).

It should be underlined that the selection of the domestic pig as an experimental animal in this investigation was on purpose. Due to relatively well-known neurochemical and physiological similarities between humans and the domestic pig, this species is considered to be an optimal animal model of processes taking place in the human nervous structures under various pathological factors (Swindle et al., 2012).
Material and methods

Chemicals

The following chemicals, antibodies and veterinary drugs were used during the present study (in alphabetical order): 0.01% NaN3, Triton x-100, 0.1% bovine serum albumin, 4% buffered paraformaldehyde (pH 7.4), 10% normal goat serum, 18% phosphate-buffered sucrose solutions, Alexa fluor 488 (donkey anti-mouse IgG, Invitrogen, Carlsbad, CA, USA), Alexa fluor 546 (Alexa fluor 546 donkey anti-rat IgG, Invitrogen), Bisphenol A (Sigma, St. Louis, USA), capsules (gelatin capsules, Carlson Laboratories, Arlington Heights, IL, USA), nNOS (anti-mouse antibody, Sigma Aldrich, Saint Louis, MS, USA, catalogue no. N218), NRG-1 (anti-rabbit antibody, Antibodies-online, Aachen, Germany, catalogue no. AA 198-229), phosphate buffer, sodium thiopental (Thiopental, Sandoz, Kundl, Austria), stressnil (Janssen, Belgium, 75 μL/kg of body weight, intramuscular), thimerosal in PBS, VIP (anti-mouse antibody, Biogenesis Inc, Poole UK, catalogue no. 9535-0504)

Experimental animals and tissue collection

This investigation was performed on 15 immature sows of the Piétrain x Duroc breed aged 8 weeks and body weight 18-20 kg. All procedures during the study were conducted according to the instruction of the Local Ethical Committee of Experiments on Animals in Olsztyn (Poland) (decision number 17/2013). The animals were divided into three groups (5 animals in each): experimental 1 (E1) group – pigs, which received capsules with bisphenol A in feed (once a day, during morning foraging) in the dose– 0.05 mg/kg body weight/day, experimental 2 (E2) group, where the animals received capsules with BPA in doses ten times higher than in the E1 group (0.5 mg/kg body weight/day) and control pigs (C group) receiving empty capsules. A dose of BPA at the level of 0.05 mg/kg body weight/day for a long period is considered to be a maximal safe dose for human. European Food Safety Authority (EFSA)
has established that this dose of BPA is a tolerable daily intake (TDI) of this substance (EFSA 2006). Nevertheless, in 2015 due to some studies describing the influence of this dose on the immune system, EFSA reduced the TDI of BPA to 4μg/kg.b.w./day (EFSA 2015). It should be pointed out that this reduction is temporary and the final decision of the EFSA depends on further toxicological studies. Moreover, according to US Environmental Protection Agency (EPA) the safety level of BPA is set during the present study at 0.05 mg/kg body weight/day (Soriano et al., 2016). Thus, the aim of the present study was to determine if BPA at a dose of 0.05 mg/kg body weight/day may change the number and neurochemical characterization of NRG-1 – positive intrahepatic nerves. In turn, the high dose of BPA used in the present study (0.5 mg/kg body weight/day) is a dose, at which clear changes in neurochemical characterization of autonomic neurons supplying other internal organs has been observed (Szymanska et al., 2018b). It is usually estimated that doses of BPA, on which humans are exposed to in everyday life are lower than the TDI used in the present study (Chen et al., 2016; Metz, 2016). Exposure of humans to BPA depends on the diet, profession and the place of residence. It is known that estimate exposure to BPA in food ranges from 0.01 to 13 μg/kg body weight/day in children and to about 4.2 μg/kg body weight/day in adults (Corrales et al., 2015). In turn the dermal daily exposure to BPA varies from 0.1 to 4.2 μg/kg body weight (Corrales et al., 2015), and the median concentration of this substance in air in factories can reach 6.67 μg/m³ (He et al., 2009). In turn, the time of BPA administration in the present experiment was estimated as a relatively short time of exposure, during which, however, changes in the neurochemical characterization of autonomic neuronal structures were observed in the previous studies (Szymanska et al., 2018b; Rytel, 2018).

After 28 days of BPA administration, all animals were subjected to euthanasia by premedication with Stressnil (intramuscular) and an overdose of sodium thiopental (given intravenously). The fragments of liver (with dimensions of approximately 2×2×2 cm., located
near the corpus of the gallbladder) were collected from all animals. Tissues were fixed in 4% buffered paraformaldehyde for 30 min., rinsed in phosphate buffer for three days and stored in 18% sucrose at 4 °C at least for three weeks. After this period, tissues were frozen at −23 °C and cut into 14 μm-thick sections using a cryostat (Microm, HM 525, Walldorf, Germany).

**Immunofluorescence method**

Sections were subjected to the routine double immunofluorescence method described previously by Szymanska et al. (2018b) using commercial primary antibodies directed towards NRG-1 (working dilution 1:1000), VIP (working dilution 1:2000) and nNOS (working dilution 1:2000). The complexes “primary antibody – antigen” were visualized using secondary antibodies conjugated with fluorochromes: Alexa fluor 488 (working dilution 1:1000) and Alexa fluor 546 (working dilution 1:1000). During the present study, the specificity of labelling was controlled by typical tests, including pre-absorption, omission and replacement tests. Fragments of the liver were evaluated under an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets.

**Evaluation of the number of NRG-1-positive nerves and statistical analysis**

The evaluation of the number of NRG-1-like immunoreactive (NRG-1-LI) fibers consisted of the counting of such fibers per observation field (0.1 mm²). Nerves were counted in five randomly selected observation fields located on six sections of the liver (fibers were counted on 30 microscopic observation fields from each animal). To prevent double-counting the same nerves, the section of the liver on which NRG-1-LI nerves were evaluated were placed at least 100 μm apart.

In turn, neurochemical characterization of nerves immunoreactive to NRG-1 was evaluated by the examination of at least 500 NRG-1-LI fibers from each animal on the simultaneous presence of VIP or nNOS. The observations were performed on at least ten
sections of the liver for each neuronal substance studied, and the number of NRG-1-LI fibers included in the experiment was considered as representing 100%. The obtained results were pooled and presented as a mean ± SEM. Statistical analysis was carried out using Student’s t test (Statistica 9.1, StatSoft, Inc., Cracow, Poland). The differences were considered statistically significant at p ≤ 0.05.

**Results**

During this investigation, the presence of NRG-1 was observed in intrahepatic nerve fibers both in physiological conditions and under the impact of BPA (Table 1, Figure 1; Figure 2). Under physiological conditions in the liver, the average number of NRG-1 – positive nerves per microscopic observation field amounted to 8.96 ± 0.41. The number of NRG-1+ fibers after administration of low doses of BPA achieved 9.63 ± 0.51 and it was not statistically significantly different from the percentage of such fibers in control animals. In turn, under high doses of BPA, the average number of NRG-1 -positive fibers per microscopic observation field amounted to 13.14 ± 0.44. BPA did not change the morphology of NRG-1-positive intrahepatic nerves. Both in the control animals and pigs treated with both doses of the toxin, such nerves were thick and clearly visible, but rather short (Figure 1, Figure 2).
Table 1. The number of NRG-1-positive intrahepatic nerve fibers and co-localization of NRG-1 with VIP and nNOS in nerves within the liver in control animals (C group) and pigs treated with low (E1 group) and high (E2 group) doses of BPA

| Nerve fibers      | Groups of animals | C group   | E1 group   | E2 group   |
|-------------------|-------------------|-----------|------------|------------|
| NRG-1 + 1)        |                   | 8.96 ± 0.41| 9.63 ± 0.51| 13.14 ± 0.44*|
| NRG-1 */VIP+ 2)   |                   | 13.03 ± 0.61| 22.34 ± 1.60*| 39.62 ± 1.89*|
| NRG-1 */nNOS+ 2)  |                   | 26.20 ± 0.90| 25.98 ± 1.67| 25.29 ± 1.83|

1) The average number of fibers in the microscopic observation field (0.1 mm²)

2) The percentage of nerves immunoreactive to VIP or nNOS with respect to all NRG-1-positive nerves (NRG-1 positive nerves were considered representing 100%)

Statistically significant (p ≤ 0.05) differences between are marked with *

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![Image of nerve fibers](image-url)
Figure 1. NGR-positive nerves (red - a) and nerves immunoreactive to VIP (green - b) in the intrahepatic nerves of control animals (I) and pigs treated with low (II) and high (III) doses of BPA. Nerves simultaneously immunoreactive to NGR-1 and VIP (yellow - c) are indicated with arrowheads

Figure 2. NGR-positive nerves (red - a) and nerves immunoreactive to nNOS (green - b) in the intrahepatic nerves of control animals (I) and pigs treated with low (II) and high (III) dose of BPA. Nerves simultaneously immunoreactive to NGR-1 and nNOS (yellow - c) are indicated with arrowheads

Both doses of BPA studied caused changes in the number of NRG-1+/VIP+ intrahepatic nerve fibers, but changes under high doses of BPA were generally more visible.
Low dose of BPA caused an increase in the average number of such nerve fibers per microscopic field to 22.34 ± 1.6% (by about 9 pp in the comparison to the control group), and high doses of this toxin - to 39.62 ± 1.89% (by 26 pp) (Table 1; Figure 1).

In turn, the percentage of NRG-1-LI intrahepatic nerve fibers simultaneously immunoreactive to nNOS amounted to 26.2± 0.9% of all NRG-1-LI fibers in the control animals, 25.98 ± 1.67% in animals treated with low doses of BPA and 25.29 ± 1.83% in pigs influenced by the high doses of the toxin. Differences between the above-mentioned values were no statistically significant (Table 1; Figure 2).

**Discussion**

During this study, the presence of NRG-1 has been shown in the nerve fibers located in the porcine liver both in the control animals and under the influence of BPA. These observations confirm the results of previous studies that NRG-1 may occur in peripheral nervous structures in various internal organs (Yue et al., 2013; Wang et al., 2014). On the other hand, it should be underlined that in spite of the fact that NRG-1 may participate in some hepatic regulatory processes connected with glucose conversion, the functions of this substance in the intrahepatic innervation still remain pure conjecture. By analogy to other organs (including central and peripheral nervous system, heart, uterus or digestive system), in which the functions of neuronal NRG-1 are better known and have been described in the previous studies (Ikeda et al., 2009; Barberán et al., 2016; Kataria et al., 2018, 2019), it can be expected that this factor participates in the regulation of the activity of ion channels located in the neuronal cells membrane and may influence the secretion and activity of neuromediators and/or neuromodulators.
Moreover, during the present study, the co-localization of NRG-1 with VIP and/or nNOS has been observed in the intrahepatic nerves. In the light of the previous investigations, it is known that neuronal factors occurring within the same nervous structures most frequently are involved in similar mechanisms (Schaible 2015). Thus, the present results suggest similar roles of NRG-1, VIP and nNOS in neuronal regulatory processes in the liver. It should be underlined that both VIP-positive, as well as nitrergic nerves have been described in the liver of various mammal species, including humans (Akiyoshi et al., 1998; Kaibori et al. 2015). Such nerves have been mainly noted around the larger portal veins, as well as near the hepatic arteries and veins, especially close to myofibroblasts, into cells, fibroblasts and endothelial cells of blood vessels (Licari et al., 2018; Kamimura et al., 2018). In turn, the presence of the above-mentioned neuronal factors in the intralobular nerve fibers is a controversial issue (Licari et al., 2018; Kamimura et al., 2018). The perivascular distribution of VIP- and/or nNOS – positive intrahepatic nerves, as well as the relatively well known significant participation of both these substances in vasodilation (Grant et al., 2006; McGarr et al., 2019) also strongly suggests that NRG-1 is also involved in regulation of the blood flow in the liver. Moreover, some previous studies have noted that both NRG-1, nNOS and VIP may participate in sensory and pain stimuli conduction (Zhang et al., 2006; Rytel and Calka 2016b). Thus, the functions of NRG-1-positive intrahepatic nerves noted during the present study cannot be excluded.

The important role of NRG-1, known from the studies on various parts of the nervous system is the participation in developmental, protective and adaptive processes (Taylor et al., 2012; Kataria et al., 2018, 2019), which is probably connected with the above-mentioned activation of Schwann cell regeneration and influence on the ion channels and secretion of neuromediators (Krishnan 2013; Viehover et al., 2001). The increase in the number of NRG-1-positive nerves under the impact of high doses of BPA noted in the present investigation
together with the previous observations, where the functions of NRG-1 in the development of the liver have been described seem to support the hypothesis that NRG-1 in intrahepatic nerves may play protective and adaptive functions. It is more likely that in the light of the previous studies it is known that the number of nervous structures immunoreactive to factors participating in neuroprotective processes increases under the impact of pathological and toxic stimuli (Rytel and Calka, 2016a, Szymanska et al., 2018b).

Moreover, co-localization of NRG-1 with VIP and/or nNOS in the same nerves, as well as BPA-induced changes in the degree of co-localization of NRG-1 and VIP, noted in the present investigation also support this theory. VIP is known as a potent neuroprotective factor increasing the survival of neuronal cells (Morell et al., 2012) and supporting blood flow of the neuronal tissue by the vasodilation (Grant et al., 2006). The neuroprotective activity of VIP is probably connected with the influence on the glial cells and stimulation of them to produce a wide range of cytokines, including IL-1α, IL-1β, IL-3 and IL-6 (Brenneman et al., 1995; Rameshwar et al., 2002). Simultaneously, VIP inhibits the activity of macrophages, which contributes to the maintenance of the homeostasis between anti- and pro-inflammatory cytokines (Fraccaroli et al., 2009). Nitric oxide (NO), like VIP, shows relaxatory effects on the vascular smooth muscles and influences the immunological system (Majewski et al., 2002), however, it is known from previous studies that it may play the role of an anti-inflammatory or a pro-inflammatory factor, depending on the type of inflammatory process and kind of inflamed tissue (Fraccaroli et al., 2009). Moreover, some investigations have described the neuroprotective functions of nitrergic neurons (Majewski et al., 2002). However, in spite of the relatively well-known functions of nitrergic innervation and NRG-1 in adaptive and protective processes, the impact of BPA on the number of NRG-1+/nNOS+ intrahepatic nerves has not been observed during the present study, which may suggest that
this population of nerves probably do not participate in mechanisms connected with the BPA–induced toxicity.

It should be underlined that the exact mechanisms and reasons for the observed changes may be connected with various processes. Most probably they result from the relatively well known neurotoxic and/or pro-inflammatory activity of BPA (Lee et al., 2013) and may be connected with disturbances in the protein synthesis and/or disorders in the intraneuronal transport of active substances from the perikaryon to the nerve endings. The observed changes may also result in the participation of NRG-1 in the sensory and pain stimuli conduction described in previous studies (Gauthier et al., 2013; Wang et al., 2014). Nevertheless, this supposition is rather unlikely because the present study has been conducted on relatively low doses of BPA and experimental animals did not show any pain symptoms.

The next hypotheses, which can explain the observed changes may be related to the fact that BPA affects the ion channels in various types of cells. Such processes have been observed even under the impact of very small concentrations of BPA and they do not result from the estrogentic activity of BPA, but from the direct connection of BPA molecules to the ion channel pore (Soriano et al., 2016). It is known that BPA may influence voltage-gated Na\(^+\), Ca\(^{2+}\) and/or K\(^+\) channels, which play crucial roles in neuronal excitability (Soriano et al., 2016). This activity may result in an increase in the number of NRG-1–positive nerves because NRG-1 is known as a factor which enhances the flow of ions across the membrane of neuronal cells and may induce functional changes in ion channels (Castillo et al., 2006).

**Conclusion**

The obtained results show that NRG-1 is present in the intrahepatic nerves and co-localized with VIP and nNOS. In turn, the influence of BPA on both the number of NRG-1–positive nerves located in the liver and the degree of co-localization of NRG-1 and VIP
suggests the participation of these nerves in mechanisms of liver degeneration and/or regeneration under the impact of BPA. Moreover, some changes in the number of NRG-1-LI nerves were observed even under the impact of low doses of BPA, which until recently has been considered to be safe for humans. The results obtained during this study seem to confirm that a BPA dose at the level of 0.05/kg bw/day is not neutral for intrahepatic innervation and the decision of EFSA on a reduction of the tolerable daily intake dose of BPA to 4 µg/kg bw/day was correct (EFSA, 2015). Changes observed in the present study are probably connected with direct neurotoxic or pro-inflammatory effects of BPA. On the other hand, due to multidirectional actions of BPA on the living organism, as well as limited knowledge about the functions of NRG-1 in the peripheral nervous system, the exact elucidation of observed changes requires further study. Nevertheless, the obtained results show that changes concerning the intrahepatic NRG-1-positive nerves may appear even under low doses of BPA and they may be the first subclinical sign of intoxication with BPA.

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