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Prenatal effects of retinoic acid on lumbar spinal cord development and liver antioxidants in rats

Janka Vašková [a], Peter Patlevič [c,*], Ladislav Vaško [a], Darina Kluchová [b]

[a] Department of Medical and Clinical Biochemistry, Pavol Jozef Šafárik University in Košice, Faculty of Medicine, Trieda SNP 1, 040 66 Košice, Slovak Republic
[b] Department of Anatomy, Pavol Jozef Šafárik University in Košice, Faculty of Medicine, Šrobárova 2, 041 80 Košice, Slovak Republic
[c] Department of Ecology, University of Presov in Presov, Faculty of Humanities and Natural Science, Ul. 17 novembra č. 1, 081 16 Prešov, Slovak Republic

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ABSTRACT

During embryonic and early postnatal development, retinoic acid (RA) regulates genes that control neuronal differentiation and neurite outgrowth from the neural tube. The effects of high levels of RA on the CNS can be detected via nitric oxide (NO), which plays a crucial role in neural transmission. The aim of the study was to investigate the prenatal influence of high levels of RA on postnatal development of nitrergic structures in lumbar spinal cord and antioxidant status. RA was administered orally at a dose of 10 mg/kg body weight to pregnant female Wistar rats during days 8–10 of gestation. Neuronal nitric oxide synthase (nNOS) of lumbar spinal cord sections was processed for visualization via nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry on postnatal day one, day twenty-one and in adults. The results suggest that prenatal administration of high levels of RA is not associated with postnatal morphological changes in nNOS-positive neurons in the rat lumbar spinal cord. An estimation of the activity of enzymes related to the storage of retinoid in the liver showed possible side effects. Suppression and deepening of superoxide dismutase activity persisted into adulthood, and a concurrent downregulation of glutathione reductase was noted. A decrease in reduced glutathione persisted until adulthood when other compensatory mechanisms were probably active to maintain an appropriate level.

Introduction

Retinoic acid (RA), a member of the retinoid family of lipids and a mediator of vitamin A activity, is an essential morphogen found in different species from invertebrates to metazoans including humans (Maden, 2008). While normal embryonic development requires retinoids, they can elicit adverse effects on the developing embryo if present in the wrong amounts, at the wrong stage or the wrong time (Sporn and Roberts, 1991). Embryos of the killifish, *Fundulus heteroclitus* exposed to low concentrations of RA (0.0001–0.1 μmol/l) develop normally, whereas those exposed to higher concentrations (0.5–100 μmol/l) develop characteristic dose-dependent defects (Vandersea et al., 1998). In mice, administration of RA on day 7 or day 8 of gestation causes retardation of general development, abnormal differentiation of the cranial neural plate and abnormal development of the hind-brain (Morriss-Kay et al., 1991). Administration of RA on day 9 of gestation induces dysmorphogenesis of the inner ear in mice (Frenz et al., 1996). Abnormalities in limb and neural plate development have been induced through RA administration between days 10 and 16 of mice gestation (Stafford et al., 1995). A study by Holson et al. (1997) found, that influence of teratogenic factors on gestation days 8, 9 and 10 of rats are critical for development of the CNS and can lead to various malformations. Therefore, in our study, we administered high doses of RA to pregnant female rats on gestation days 8, 9 and 10.

Retinoids are involved in the mechanisms underlying the inflammatory response, and have been related to the generation and expression of nitric oxide (NO; Seguin-Devaux et al., 2002), prostaglandins (Devaux et al., 2001) and cyclooxygenases (Li et al., 2002). In addition to the widely known beneficial processes where NO plays a role (including neurotransmission, blood pressure regulation, immunomodulation), under certain conditions production of NO (as well as other NOS-derived oxidants) can be

Abbreviations: GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; IML, intermediolateral nucleus; NADPH-d, nicotinamide adenine dinucleotide phosphate-diaphorase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; RA, retinoic acid; SOD, superoxide dismutase.

* Corresponding author.
E-mail address: peter.patlevic@unipo.sk (P. Patlevič).

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detrimental owing to induced nitrosation and oxidative stress (Wink and Mitchell, 1998) or altered enzyme activities; for example P450-mediated drug metabolism (Miller, 2004).

A family of three nitric oxide synthase 1 (NOS) isoforms produces nitric oxide (NO) and L-citrulline via a stepwise oxidation of the guanidinium nitrogen of L-arginine. Although all three NOS isoforms (two constitutively expressed isoforms; neuronal (nNOS) and endothelial (eNOS) and one inducible isoform (iNOS)) produce identical products, the function of NO varies widely in terms of physiological functions due to the varying localization of the isoforms within different cell populations of the body (Miller, 2004). Through NO production, the enzyme is involved in the promotion of axonal elongation and regulation of growth cone advancement (Van Wagenen and Rehder, 1999), and also causes cell death in the nervous system during development due to nitrosative stress (Freire et al., 2009). The histochemical reaction for NADPH-diaphorase (NADPH-d) colocalizes with neuronal NOS (nNOS), therefore the localization of NADPH-d is generally considered to be reflective of the presence of nNOS and is used as a specific histochemical marker for NO containing neurons (Kaur et al., 1999). In the spinal cord, NADPH-d activity has been detected in the dorsal horn, around the central canal, and at the intermediolateral cell column of the spinal cord (Xu et al., 2006).

The liver is the principal organ responsible for the storage of retinol (Inder et al., 1999). The embryo is unable to synthesize retinol and is strongly dependent on the maternal delivery of retinol itself or precursors such as retinyl esters (retinyl palmitate) or carotenoids. Furthermore, the teratogenic effects of RA seem to appear about day 8 of gestation, possibly extending until day 12, during the initiation of abdominal formation (Quemelo et al., 2007). Retinol carrier molecules are present during this period, not only in the maternal placental decidua basalis, but also in the embryo from day 7 in the embryonic yolk sac and from day 11.5 post-coitum in the liver (Wendler et al., 2003).

Administration of vitamin A and its derivatives in excess can lead to fibrosis and hepatocellular dysfunction (Choudhary and Swami, 2012). The toxicity mechanisms are poorly understood, but modulation of the hepatic function by vitamin A supplementation does affect the liver mitochondria structure and function (Leo and Lieber, 1999). Cellular responses to RA *in vitro* ranged from cell death to cell differentiation (Huang et al., 2006).

We found no research studies that examined morphological changes of nitrergic structures in the lumbar spinal cord after prenatal influence of teratogenic RA. Our study focused on the NADPH-d/nNOS, around the central canal, dorsal horn and at the intermediolateral (IML) nucleus of the rat lumbar spinal cord until adulthood to assess if normal development was affected during this critical 3-day period of pregnant female rat overdose. Increased availability and utilization of RA by the embryo itself leads to the question of how the excess supply of RA could be used by the embryo, and the possibility that deviations from normal metabolic conditions might lead to the induction of oxidative stress in the retinol storage organ. Therefore, we also measured the activities of selected enzymatic and non-enzymatic antioxidants in liver mitochondria in the offspring of treated females.

Materials and methods

Animals

The study was carried out on the lumbar spinal cords and livers of Wistar rats of both sexes at postnatal day one, day twenty-one and in adults. The rats were kept under standard conditions, subject to inspection from the Ethical Commission of the Medical Faculty, University of Pavol Jozef Šafárik in Košice, Slovak Republic. The experiment was conducted in accordance with the "European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (86/609/EU) and with the approval of the State Veterinary and Food Administration of the Slovak Republic (No. 1696/07-221a). The rats had free access to food and drinking water.

Retinoic acid administration

Retinoic acid (R7632; Sigma, Schelldorf, Germany) was administered orally to 12 adult female rats at a dose of 10 mg/kg body weight (BW) on days 8, 9, and 10 of gestation (RA group) based on an earlier study (Holson et al., 1997). The control group consisted of 12 adult female rats given olive oil orally for the same period.

Tissue processing

At postnatal day one (P1), day twenty-one (P21) and in adults (P90), the experimental animals (8 animals per group, at P21 and P90 of both sexes in a 1:1 ratio, while individuals still came from the same litters) were deeply anesthetized with thiopental [50 mg/kg (i.v.)] and perfused transcardially with 500 ml of heparinized 0.9% saline solution, followed by 2000 ml of freshly prepared 4% paraformaldehyde (Sigma) + 0.1% glutaraldehyde buffered with 1 M sodium phosphate (pH 7.4). The lumbar spinal cord was removed, postfixed for 2 h in 4% paraformaldehyde, and then placed in 30% sucrose. Frozen horizontal segments of the lumbar spinal cord were sectioned at a thickness of 35 μm using a cryostat. Sections were collected in 0.1 M phosphate buffer (PBS) (pH 7.4) for histochemical processing.

NADPH-d histochemistry

NADPH-d was detected with the indirect method modified from Scherer-Singler et al. (1983) as follows: free floating sections were incubated for 1 h at 37 °C in a solution containing 1 mg/ml of nitroblue tetrazolium (NBT; Sigma), 0.5 mg/ml β-NADPH (Sigma), and 1.25 mg/ml monosodium malate salt (Sigma) dissolved in 0.1 M PBS (pH 8.0), and 0.8% Triton X-100. Control sections were incubated in substrate-free media. Sections were monitored every 20 min to avoid overstaining. Following the reaction, the sections were rinsed in 0.1 M PBS (pH 7.4), mounted on slides, air-dried overnight, coverslipped with Entellan (Merck, Darmstadt, Germany) and examined by light microscopy. Micrographs of sections were taken by microscope Optika, model B600 Ti with digital camera system Moticam 2300. As a communication program between the camera and PC was used Motic Image Plus software, version 2.0 ML.

Antioxidant enzyme activity

The livers of anesthetized rats were removed and placed in an isolation medium containing 320 mM/l sucrose, 10 mM/l Tris and 1 mM/l EDTA (pH 7.4). Mitochondria were isolated from livers using the method of Fernández-Vizzara et al. (2010). Protein concentration was determined according to bicinchoninic acid (BCA) assay (Smith et al., 1985) and expressed as milligrams protein per milliliter of homogenate (mg prot/ml). Bovine serum albumin was used as a standard. Glutathione reductase activity (GR, E.C.1.6.4.2) was measured by the method described by Carlberg and Mannervik (1985) and the activity of glutathione peroxidase (GPx, E.C.1.11.1.9) by the method according to Zagrodski et al. (1998). Superoxide dismutase activity (SOD, E.C.1.15.1.1) was measured using an SOD-Assay Kit-WST (Fluka, Buchs, Switzerland) and that of reduced glutathione (GSH) was determined by the method of Floreani et al. (1997) using Ellman's reagent. An M 501 Single beam