Ecotoxicology and Environmental Safety 113 (2015) 314–320

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Direct effects of the algal toxin, domoic acid, on ovarian function: Bovine granulosa and theca cells as an *in vitro* model

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ARTICLE INFO

Article history:
Received 20 August 2014
Received in revised form
27 November 2014
Accepted 2 December 2014
Available online 18 December 2014

Keywords:
Domoic acid
Steroidogenesis
Cell proliferation
In vitro effects

ABSTRACT

Domoic acid (DA) is a potent neurotoxin produced by alga *Pseudo-nitzschia* spp. and has been associated with reproductive disorders in mammals. The aim of this study was to investigate if DA can affect the reproductive system via direct action on ovarian function. Bovine granulosa and theca cells were used as in vitro models for evaluating DA effects on ovarian cell proliferation and steroid production. In small-follicle granulosa cells (SMGC), cell proliferation and estradiol (E2) production was not affected ($P > 0.05$) while progesterone (P4) production was inhibited ($P < 0.05$) by DA at all doses tested. In large-follicle granulosa cells (LGGC), DA had no effect ($P > 0.05$) on cell proliferation or P4 production while E2 production was stimulated by 1 and 5 μg/ml DA ($P < 0.05$). DA (1 μg/ml) attenuated ($P < 0.05$) insulin-like growth factor 1 (IGF-1)-induced P4 production by large-follicle theca cells (LGTC), but did not affect androstenedione (A4) production or proliferation of LGTC. In glutamate-free medium, DA inhibited ($P < 0.05$) SMGC E2 production and this inhibition was similar to inhibition of E2 by trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid monohydrate (ACPD; a selective metabotropic glutamate receptor subtype agonist) while kainic acid (KA; an ionotropic glutamate receptor subtype agonist) had no effect ($P > 0.10$) on E2 production. Collectively, these results show for the first time that DA has direct effects on ovarian GC and TC steroidogenesis. Because DA inhibited E2 and P4 production, DA has the potential to be an endocrine disruptor.

1. Introduction

Domoic acid (DA) is an amino acid produced by *Pseudo-nitzschia* spp., a cosmopolitan genus of pennate diatoms restricted to the marine environment (Mos, 2001). DA, structurally an analog of the neurotransmitter L-glutamate, is a non-N-methyl-D-aspartate (NMDA) receptor agonist, and shows affinity for both kainic acid (KA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Hampson et al., 1992; Berman and Murray, 1997; Hampson and Manalo, 1998). A synergic action of DA, acting at AMPA receptors, with other neurotoxic excitatory amino acids (EAA), such as glutamic acid and aspartic acid, normally present in high amount in DA-contaminated mussels, has been previously reported (Novelli et al., 1992; Giordano et al., 2006). Neurotoxicity of purified DA was lower compared with those obtained using extracts of DA-contaminated mussels, suggesting that other factors are involved (Novelli et al., 1992). Indeed DA, binding to KA and AMPA receptors, stimulates release of endogenous EAA which activate NMDA receptors and allow the increase of cytoplasmic calcium leading to final neuronal injury (Berman and Murray, 1997; Berman et al., 2002; Giordano et al., 2006).

In humans, DA-contaminated shellfish consumption (Costa et al., 2010) causes a syndrome called Amnesic Shellfish Poisoning (ASP), characterized by the presence of neurological signs (Lefebvre and Robertson, 2010). In order to protect the public health, a limit of 20 mg DA/kg shellfish meat has been imposed in the European Union area (European Food Safety Agency (EFSA), 2008) and in other countries (Canada, USA, New Zealand and Australia) (Costa et al., 2010). It has been demonstrated that DA can affect

Abbreviations: DA, domoic acid; GC, granulosa cells; TC, theca cells; SMGC, small-follicle granulosa cells; LGGC, large-follicle granulosa cells; LGTC, large-follicle theca cells; GH, growth hormone; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ACPD, trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid monohydrate; NMDA, N-methyl-D-aspartate; EAA, excitatory amino acids; CNS, central nervous system; E2, estradiol; KA, kainic acid; P4, progesterone; A4, androstenedione; IGF-1, insulin-like growth factor 1; FSH, follicle stimulating hormone; LH, luteinizing hormone; FCS, fetal calf serum; RIA, radioimmunoassay; SEM, standard error of the mean; SAS, Statistical Analysis System; GI, gastro-intestinal

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http://dx.doi.org/10.1016/j.ecoenv.2014.12.009
0147-6513/

organs other than the central nervous system (CNS) (Perl et al., 1990; Tryphonas et al., 1990a,1990b; Costa et al., 2010), moreover DA may also have indirect effects on hormone production such as thyroid-stimulating hormone (Arufe et al., 1995, 2002) and adrenocorticotropic hormone (Perry et al., 2009; Gulland et al., 2012).

Previous studies (Dakshinamurti et al., 1993; Levin et al., 2005; Tanemura et al., 2009) demonstrated that pre-natal DA exposure leads to alterations in the developmental process in rodents. Tiedeken et al. (2005) found that eggs of *Danio rerio* (zebrafish) exposed to doses ranging from 0.12 to 17 μg DA g^{-1} egg weight showed a reduction in hatching, and the embryos hatching at 4 days post-fertilization were devoid of touch reflexes. DA can be detected in milk, indicating the possibility of a risk for neonatal exposure (Maucher and Ramsdell, 2005). In California Sea Lions, some cases of reproductive symptoms are recorded after DA poisoning (Brodie et al., 2006; Ramsdell and Zabka, 2008; Goldstein et al., 2009). In particular, Brodie et al. (2006) suggested that DA had a role in causing reproductive failure in pregnant female California Sea Lions through abortion or premature parturition of pups. Because DA is able to cross the placenta, the fetus is exposed to DA (Goldstein et al., 2009). However, direct or indirect effects of DA on the fetus, placenta or ovary are unclear, as well as the mechanism involved in these effects.

Because DA is able to cause reproductive disorders (Brodie et al., 2006; Ramsdell and Zabka, 2008; Goldstein et al., 2009), the present study was performed in order to clarify whether DA directly affects ovarian function. Ovarian steroids are critical for uterine function, such as establishment and maintenance of the pregnancy (Wood and Strauss, 2002; Lonergan and Forde, 2014); therefore, an alteration in the production of these hormones may lead to abortion and infertility (Walsh et al., 2011). Granulosa cells isolated from bovine ovarian follicles and cultured in vitro have been previously used as a reliable in vitro model for reproductive toxicology research, and this in vitro system has been successfully applied for determining the effects on steroidogenesis caused by contaminants (Mlynarczuk et al., 2009; Petro et al., 2012). In this study we evaluated the in vitro effects of DA on cell proliferation and steroid production using primary bovine ovarian cells. In particular, we utilize a cell culture system that is highly responsive to hormones such as insulin-like growth factor-1 (IGF-1), known to induce proliferation and steroidogenesis in gonadotropin-stimulated bovine ovarian cells (Stewart et al., 1995; Spicer and Chamberlain, 1998; Spicer et al., 2002).

2. Materials and methods

2.1. Cell isolation and culture

Ovaries from non-pregnant Angus beef cows were collected from a commercial slaughterhouse and transported (< 120 min) to the laboratory on ice. The slaughterhouse facilities were in accordance with the USDA/APHIS/Animal Care Code of Federal Regulations, Terrestrial Animal Health Standards Commission Report, Chapter 7.5 otherwise known as “Slaughter of Animals”. The ovaries were washed three times in saline (0.15 NaCl), immersed in 70% ethanol for 30 s, and then washed again three times with saline. The ovaries were kept on ice in saline until the GC were collected (Langhout et al., 1991; Spicer et al., 1993). GC from small (1–5 mm; SMGC) and large (8–22 mm; LGGC) follicles were isolated as previously described (Langhout et al., 1991; Spicer and Chamberlain, 1998; Lagaly et al., 2008). This size classification was based on previous studies showing that SMGC are less responsive to follicle-stimulating hormone (FSH) and insulin-like growth factor 1 (IGF-1) than are LGGC (Spicer and Chamberlain 1998; Spicer et al., 2002) and that large follicles have much greater estradiol (E2) concentrations than small follicles (Stewart et al., 1996; Spicer and Aad, 2007). Theca cells (LGTC) were collected from large (8–22 mm) follicles as previously described (Stewart et al., 1995; Spicer and Chamberlain, 1998; Lagaly et al., 2008).

GC were recovered by centrifugation at 291 × *g* for 10 min and were washed two times with 7 ml of serum-free medium; at each wash, cells were separated from medium via centrifugation (291 × *g* at 4 °C for 5 min). After the last centrifugation the supernatant was aspirated and replaced with 2 ml of enzyme containing medium (0.5 mg/ml of DNase and 1.25 mg/ml of collagenase) to prevent clumping of cells as previously described (Spicer et al., 2002; Aad et al., 2006). Numbers of viable cells were determined using the trypan blue exclusion method (Langhout et al., 1991; Spicer et al., 1993; Tiemann et al., 2003). Viability of SMGC and LGGC averaged 70% and 87%, respectively, and is within the range of viabilities previously reported for these cell types collected from slaughterhouse tissues (Langhout et al., 1991; Spicer et al., 2002). Viable cells (1.5×10^5 in 20–80 μl of medium) were plated on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 ml of basal medium [1:1 Dulbecco modified Eagle medium (DMEM) and Ham F-12 medium] containing 10% fetal calf serum (FCS), gentamycin (0.12 mM), glutamine (2.0 mM), and sodium bicarbonate (38.5 mM). Cultures were kept at 38.5 °C in a humidified 95% air and 5% CO_2 environment and medium was changed every 24 h. To obtain an optimal attachment, cells were maintained in the presence of 10% FCS for the first 48 h of culture. After this time, GC were washed twice with serum-free medium and the various treatments applied in serum-free medium containing 500 ng/ml testosterone (as an E2 precursor) for 48 h with a medium change at 24 h. The doses of DA tested were: 0, 1, 5, and 20 μg/ml to evaluate the effect of DA on steroid production by SMGC (Exp. 1) and LGGC (Exp. 2). Culture medium (1 ml per well) was supplemented with FSH at 30 ng/ml, IGF-1 at 30 ng/ml and testosterone at 500 ng/ml. Doses of FSH and IGF-1 were selected based on previous studies (Spicer et al., 2002; Lagaly et al., 2008). Both FSH and IGF-1 were added to all treatments because either hormone alone has little or no effect on GC E2 production (Spicer et al., 2002).

After aspiration of follicular fluid, large follicles were bisected and GC were separated from the TC via blunt dissection and the theca interna was enzymatically digested as previously described (Stewart et al., 1995; Spicer and Chamberlain, 1998; Aad et al., 2006). By using sterile syringe filter holders with metal screens of 149 μm mesh (Gelman, Ann Arbor, MI), the non-digested tissue was eliminated, and filtered TC were then centrifuged at 50 × *g* for 5 min. LGTC were washed with serum-free medium and resuspended in serum-free medium containing collagenase and DNase as described for GC. LGTC (2.0×10^5 viable cells/well) were plated and cultured as described for GC. Viability of LGTC averaged 99%. The doses of DA tested were: 0, 0.3, 1, and 5 μg/ml in the presence or absence of 30 ng/ml of IGF-1 (Exp. 3). Culture medium was also supplemented with 30 ng/ml of luteinizing hormone (LH) because progesterone (P4) and androstenedione (A4) production are not induced by IGF-1 in the absence of LH whereas LH alone increases A4 production (Stewart et al., 1995; Spicer and Stewart, 1996).

To compare the effect of DA with other agonists of glutamate receptors (Exp. 4), SMGC were treated for 48 h with 1 μg/ml of DA in the absence and presence of 1 μg/ml of glutamate. Also tested were 25 μg/ml of either kainic acid (KA), an ionotropic glutamate receptor subtype agonist (Wisden and Seeburg, 1993), or trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid monohydrate (ACPD), a selective metabotropic glutamate receptor subtype agonist (Palmer et al., 1989). Dose of KA and ACPD were selected based on previous studies in other cell types (Schoepp et al., 1991; 1996; Domin et al., 2014). For all treatments, culture medium was