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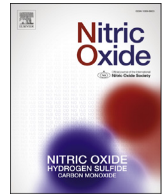

Endogenously produced hydrogen sulfide is involved in porcine oocyte maturation *in vitro*

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ABSTRACT

Hydrogen sulfide, one of three known gasotransmitters, is involved in physiological processes, including reproductive functions. Oocyte maturation and surrounding cumulus cell expansion play an essential role in female reproduction and subsequent embryonic development. Although the positive effects of exogenous hydrogen sulfide on maturing oocytes are well known, the role of endogenous hydrogen sulfide, which is physiologically released by enzymes, has not yet been described in oocytes. In this study, we observed the presence of Cystathionine β-Synthase (CBS), Cystathionine γ-Lyase (CTH) and 3-Mercaptopyruvate Sulfurtransferase (3-MPST), hydrogen sulfide-releasing enzymes, in porcine oocytes. Endogenous hydrogen sulfide production was detected in immature and matured oocytes as well as its requirement for meiotic maturation. Individual hydrogen sulfide-releasing enzymes seem to be capable of substituting for each other in hydrogen sulfide production. However, meiosis suppression by inhibition of all hydrogen sulfide-releasing enzymes is not irreversible and this effect is a result of M-Phase/Maturation Promoting Factor (MPF) and Mitogen-Activated Protein Kinase (MAPK) activity inhibition. Futhermore, cumulus expansion expressed by hyaluronic acid (HA) production is affected by the inhibition of hydrogen sulfide production. Moreover, quality changes of the expanded cumuli are indicated. These results demonstrate hydrogen sulfide involvement in oocyte maturation as well as cumulus expansion. As such, hydrogen sulfide appears to be an important cell messenger during mammalian oocyte meiosis and adequate cumulus expansion.

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1. Introduction

Meiosis in mammalian oocytes is spontaneously arrested at the dictyotene of prophase I. Prior to this, growing oocytes synthesise large amounts of protein essential for the resumption of this meiotic block, which is followed by oocyte meiosis [1]. A fully-grown dictyate oocyte, often called a germinal vesicle (GV) oocyte, holds the meiotic block until gonadotropin stimulation *in vivo* and *in vitro* conditions, when the re-initiation of oocyte meiosis is manifested as germinal vesicle breakdown (GVBD). GVBD is followed by further stages of meiosis I and the establishment of the second meiotic block at metaphase II (MII). The process that begins with GVBD and continues through meiosis I to MII is called meiotic maturation. Oocyte maturation is necessary for fertilisation ability and successful embryonic development [2]. Therefore, oocyte maturation is a key factor in female fertility as well as in

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assisted reproductive therapy.

Cumulus cells surround the oocyte, and together, they create a cumulus–acolyte complex (COC). Oocyte maturation is accompanied by an increased production of extracellular matrix compounds in the cumulus cells mass [3,4]. COC enlargement associated with mucification, known as cumulus expansion, is based on the production of glycosaminoglycans, especially hyaluronic acid [5,6]. Expanded cumuli prevents the flow of certain meiosis inhibiting factors to the oocyte [7,8]. Moreover, HA itself acts as a signal molecule regulating oocyte maturation [9,10]. These changes result in the activation of key cell cycle kinases that are pivotal for oocyte maturation: M-Phase/Maturation Promoting Factor (MPF) and Mitogen-Activated Protein Kinase (MAPK) [11,12]. The ways in which up-stream factors regulate MPF/MAPK-driven oocyte maturation are not yet fully understood. Based on our recent observations, a potent group of gasotransmitters, gaseous molecules with second messenger action, are evidently involved in the regulation of oocyte metabolism [13,14].

Of the three described gasotransmitters – nitric oxide, hydrogen sulfide and carbon monoxide, only the role of nitric oxide has been more detail studied in oocyte maturation and cumulus expansion [summarised in Ref. [15]]. In addition to nitric oxide, endogenously released hydrogen sulfide is also required for the regulation of many cell functions [summarised in Refs. [16,17]]. However, the role of hydrogen sulfide in oocyte maturation and cumulus expansion remains unclear. Nevertheless, the accelerational effect of sodium sulfide, an exogenous hydrogen sulfide donor, on MPF/MAPK activity and oocyte maturation was recently described. Moreover, this hydrogen sulfide donor also influences hyaluronic acid (HA) production and cumulus expansion [13]. This indicates that endogenous production of hydrogen sulfide may play a physiological role in the regulation of oocyte maturation and cumulus expansion.

Endogenously releasing hydrogen sulfide from amino acid L-cysteine is catalysed by pyridoxal phosphate-dependent enzymes: Cystathionine β-Synthase (CBS), Cystathionine γ-Lyase (CTH) and/or 3-Mercaptopyruvate Sulfurtransferase (3-MPST) [18,19]. CBS is the only hydrogen sulfide-releasing enzyme to be observed in ovarian follicles [20,21]. Neither the physiological production of hydrogen sulfide nor the presence of CTH and 3-MPST has been indicated in oocytes.

We hypothesise that three hydrogen sulfide-releasing enzymes, CBS, CTH and 3-MPST, are present in mammalian oocytes and produce endogenous hydrogen sulfide, which is involved in the regulation of oocyte maturation and cumulus expansion. The aims of the present study consisted of the following: 1) to detect mRNA for CBS, CTH and 3-MPST in pig oocytes and cumulus cells; 2) to localise CBS, CTH and 3-MPST proteins in pig oocytes during oocyte maturation; 3) to demonstrate the effects of inhibitors of these hydrogen sulfide-releasing enzymes on pig oocyte maturation; 4) to measure MPF and MAPK activity in oocytes; and 5) to evaluate cumulus expansion based on HA production.

2. Materials and methods

2.1. Oocyte isolation and in vitro maturation

Porcine ovaries were obtained from 6- to 8-month-old noncycling gilts (a crossbreed of Landrace x Large White), at the local slaughterhouse (Jatky Plzen a.s., Plzen, Czech Republic) and kept at 39 °C until arrival to the laboratory. Cumulus-oocyte complexes (COCs) were collected from ovarian follicles with a diameter of 2–5 mm by a 20-gauge aspirating needle. Only fully grown oocytes with intact cytoplasm, surrounded by compact cumuli, were selected for experiments.

The COCs were matured in a modified M199 medium (Sigma–Aldrich, USA) supplemented with 32.5 mM sodium bicarbonate, 2.75 mM calcium L-lactate, 0.025 mg/ml gentamicin, 6.3 mM HEPES, 13.5 IU eCG: 6.6 IU hCG/ml (P.G.600; Intervet International B.V., Boxmeer, Holland) and 5% (v/v) foetal bovine serum (Sigma–Aldrich, USA). The culture medium contained oxamic acid, DL-propargylglycine and/or α-ketoglutaric acid, specific inhibitors of CBS, CTH and 3-MPST, respectively, in double- or triple-combination. An effective concentration of the triple-combination of inhibitors (2 mM oxamic acid, 2 mM DL-propargylglycine and 5 mM α-ketoglutaric acid dissolved in M199 medium; $3C_i$) was used for subsequent experiments. The COCs were matured for 16–48 h in 4-well Petri dishes (Nunc, Fisher Scientific, USA) containing 1.0 ml of culture medium, at 39 °C in a mixture of 5.0% CO_2 in air.

2.2. Assessment of oocyte meiotic maturation

At the end of culture, the COCs were treated with 0.1% bovine testicular hyaluronidase (Sigma–Aldrich, USA) dissolved in M199 medium and cumulus cells were separated from oocytes by repeated pipetting through a narrow glass pipette. Subsequently, the oocytes were mounted on microscope slides with vaseline, covered with a cover glass, and fixed in ethanol–acetic acid (3:1, v/v) for at least 48 h. The oocytes were stained with 1.0% orcein in 50% aqueous-acetic acid and examined under a phase contrast microscope. Five groups of meiotic maturation stages were determined in accordance with the published criteria by Motlik and Fulka [22]: GV – germinal vesicle, LD – late diakinesis, MI – metaphase I, AITI – anaphase I to telophase I transition, MII – metaphase II.

2.3. Real time RT-qPCR analysis

The samples for quantitative Real Time RT-qPCR analysis of CBS, CTH and 3-MPST mRNAs were prepared from growing oocytes, fully grown immature (GV), maturing (MI) and matured (MII) oocytes. Concurrently, cumulus cells were used for the same analysis.

RNA was isolated using a NucleicAcid PrepStation 6100 (Applied Biosystems, Fisher Scientific, USA) in accordance with the instruction manual. Total mRNA was transcribed to cDNA with a High-Capacity cDNA Achieve kit (Applied Biosystems, USA) in accordance with manufacture instructions. cDNA was synthesised in a final volume of 100 μl. Sets of specific primers were synthesised in accordance with known sequences to amplify specific products for CBS, CTH and 3-MPST (Table 1).

Real-time PCR was performed using a standard Taq-Man PCR kit protocol (Applied Biosystems, USA). Each PCR reaction was performed in triplicate in a total volume of 10 μl with a 500 nM gene-specific primer and 200 nM TaqMan MGB probes, 5 μl of 2× concentrated Fast TaqMan Universal Master Mix (Applied Biosystems, USA), 1 μl cDNA, and nuclease-free water up to volume. The 7500 Fast Real-Time PCR System (Life Technologies, USA) was utilised for RT-qPCR reactions and the programme used was as follows: 95 °C for 20 s followed by 40 cycles of 95 °C for 2 s and 60 °C for 20 s.

The relative quantification of mRNA expression for each enzyme was determined with data from SDS software using the arithmetical formula $2^{-\Delta\Delta CT}$, according to the comparative Ct method [23], representing the amount of target, normalised to the GAPDH endogenous control as reference [24] and related to fully grown GV oocytes and their cumulus cells.

2.4. Immunocytochemistry and image analysis

GV, MI and MII oocytes were denuded from cumulus cells, fixed and processed as early described Yi et al. [25]. Briefly, oocytes were

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