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Original Contribution

Nitric oxide synthase activity is critical for the preovulatory epidermal growth factor-like cascade induced by luteinizing hormone in bovine granulosa cells

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ABSTRACT

In rabbits and rodents, nitric oxide (NO) is generally considered to be critical for ovulation. In monovulatory species, however, the importance of NO has not been determined, nor is it clear where in the preovulatory cascade NO may act. The objectives of this study were (1) to determine if nitric oxide synthase (NOS) enzymes are regulated by luteinizing hormone (LH) and (2) to determine if and where endogenous NO is critical for expression of genes essential for the ovulatory cascade in bovine granulosa cells in serum-free culture. Time– and dose–response experiments demonstrated that LH had a significant stimulatory effect on endothelial NOS (NOS3) mRNA abundance, but in a prostaglandin-dependent manner. NO production was stimulated by LH before a detectable increase in NOS3 mRNA levels was observed. Pretreatment of cells with the NOS inhibitor L-NAME blocked the effect of LH on the epidermal growth factor (EGF)-like ligands epiregulin and amphiregulin, as well as prostaglandin–endoperoxide synthase-2 mRNA abundance and protein levels. Similarly, EGF treatment increased mRNA encoding epiregulin, amphiregulin, and the early response gene EGR1, and this was inhibited by pretreatment with L-NAME. Interestingly, pretreatment with L-NAME had no effect on either ERK1/2 or AKT activation. Taken together, these results suggest that endogenous NOS activity is critical for the LH-induced ovulatory cascade in granulosa cells of a monotocous species and acts downstream of EGF receptor activation but upstream of the EGF-like ligands.

Ovulation is the process of rupture of the ovarian follicle that leads to the release of an oocyte into the oviduct for fertilization. Current estimates are that approximately 20% of cases of infertility in women are related to problems of ovulation [1], and 10% of infertility is explained by failure to ovulate in economically important species such as cattle [2].

The ovulatory process is initiated by a surge of LH that acts upon LH receptors present on the surface of follicular granulosa cells. This leads to activation of proteolytic sheddase enzymes that cause the release of the membrane-bound proteins epiregulin (EREG) and amphiregulin (AREG), which are members of the epidermal growth factor (EGF) family. These proteins activate the EGF receptor on granulosa and cumulus cells, via a mitogen-activated protein kinase pathway, to stimulate the expression of prostaglandin–endoperoxide synthase 2 (PTGS2, also known as COX2) as well as AREG and EREG [3–6]. The enzyme PTGS2 is key to the production of prostaglandins from arachidonic acid, and prostaglandins are believed to regulate the expression/activity of proteases such as plasminogen activator that degrade the follicle wall and result in follicle rupture [7].

Although LH is the main trigger for ovulation, other locally acting factors are known to be active. One of these is nitric oxide (NO), which has been shown to stimulate ovulation in rodents and rabbits. Nitric oxide is generated by NO synthase (NOS) enzymes, two of which, inducible NOS (NOS2) and endothelial NOS (NOS3), have been well described in the ovarian follicle [8–11]. Whether neuronal NOS (NOS1) is important to ovarian function is not certain, as it is reported to be expressed at very low levels if at all [8]. Treatment with NOS inhibitors blocked human chorionic gonadotrophin (hCG)-induced ovulation in rats [12–14] and rabbits [15], and the loss of the NOS3 gene negatively affected ovulation rate in mice [16]. The endogenous NOS/NO system is activated during ovulation, as evidenced by increased NOS3 mRNA/protein levels [8–10] and NO production [15,17] in follicular cells induced by an ovulatory dose of hCG. The involvement of NOS2 is not certain, as Van Voorhis et al. and Faletti et al. reported decreased levels of NOS2 mRNA/protein in the follicle after hCG treatment [8,10], whereas Jablonka-Shariff et al. reported an

Abbreviations: LH, luteinizing hormone; EREG, epiregulin; AREG, amphiregulin; EGF, epidermal growth factor; EGR1, early growth response-1; PTGS2, prostaglandin–endoperoxide synthase-2

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increase in NOS2 protein levels [9]. NO induces the ovulatory process through an increase in prostaglandin secretion; inhibition of NO production reduced prostaglandin secretion, and the administration of NO donors stimulated prostaglandin synthesis in rats and rabbits [15,17], at least in part by altering PTGS2 activity [18]. It is not known if NO acts directly on PTGS2 expression or further upstream (AREG/EREG), or if the regulation of NOS3 expression is a direct effect of LH or mediated by downstream factors such as EGF receptor (EGFR) activation or prostaglandin secretion.

The importance of NO for ovulation in monovulatory species has not been established although it has been demonstrated that hCG increases granulosa cell NOS3 mRNA levels in sheep [19]. In horses, hCG increased follicular fluid NO content [20], and inhibition of NOS activity in vivo slowed follicle growth and delayed (but did not block) ovulation [21].

In this report, we set out to investigate the role of NOS in ruminants using a bovine granulosa cell culture system in which AREG, EREG, and PTGS2 mRNA levels are regulated by LH [22] in a manner similar to that observed in vivo [23]. The specific aims of the study were to examine the importance of NO in the ruminant preovulatory cascade; to determine if NOS enzymes are regulated by LH, EGF, or prostaglandin signaling; and to identify the locus of action of NO in the preovulatory cascade.

Materials and methods

Cell culture

The granulosa cell culture was as previously described [22], in which EREG, AREG, and PTGS2 mRNA levels are acutely upregulated by LH. The reagents were obtained from Invitrogen except where otherwise stated. Bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir and were transported to the laboratory in phosphate-buffered saline (PBS) containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and Fungizone (1 μg/ml), at 35 °C. Five to eight ovaries that each contained a single large follicle (> 10 mm diameter) were selected for each replicate. Cells were collected from the large follicle by aspiration, pooled, and washed twice by centrifugation at 219g for 20 min each. Cell viability was estimated with 0.4% trypan blue stain. Cells were seeded into 24-well tissue culture plates (Sarstedt) at a density of 1×10^6 viable cells per well in 1 ml Dulbecco's modified Eagle's medium (DMEM)–F12 supplemented with sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), bovine serum albumin (0.1%; Sigma–Aldrich), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), nonessential amino acid mix (1.1 mM), androstenedione (10^{-7} M), follicle-stimulating hormone (1 ng/ml), insulin (10 ng/ml), and 2% fetal calf serum (Hyclone). Cultures were maintained at 37 °C in 5% CO_2 for 24 h. The medium was then replaced with 0.9 ml serum-free DMEM–F12 with antibiotics for 18 h, at which point the experimental treatments were added in 100 μl DMEM–F12 (with antibiotics) as described below.

Study 1: regulation of NOS mRNA abundance and activity in granulosa cells

To determine the regulation of NOS mRNA abundance, time– and dose–response studies were performed with bovine LH (AFP11743B; National Institute of Diabetes and Digestive and Kidney Diseases, NIH); cells were stimulated with 400 ng/ml for 0, 1, 3, 6, or 12 h or for 12 h with 0, 10, 100, or 400 ng/ml LH. A further dose–response study was conducted with recombinant human EGF (R&D Systems) at 0, 1, 10, or 100 ng/ml for 6 h. To assess the potential role of prostaglandins in NOS expression, prostaglandin E2 (PGE2; Sigma–Aldrich) or prostaglandin F2α (PGF2α; Sigma–Aldrich) was added at 0, 1, or 10 μM for 6 h. In subsequent experiments, cells were pretreated for 2 h with the prostaglandin synthesis inhibitor indomethacin (Sigma–Aldrich) before challenge with EGF (5 ng/ml); this dose of EGF was chosen based on the EGF dose response. At the end of treatment, the culture medium was removed and TRIzol (Invitrogen) added to the wells for RNA extraction.

Intracellular NO production was assessed with the fluorescent NO-sensitive dye 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) essentially as described [24]. Cells were cultured as described above and pretreated with DAF-FM DA (10 μM) 2 h before the addition of LH (400 ng/ml) or EGF (5 ng/ml). The cells were examined under an Olympus FV1000 laser-scanning confocal microscope at times 0 and 180 min. Digital images were captured from the same field and fluorescence intensity (ImageJ software) was quantified.

Study 2: effect of NO on the preovulatory cascade in granulosa cells

To determine the role of NO in the preovulatory cascade, we chose to inhibit endogenous NO production by the addition of the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma–Aldrich). Cells were pretreated with 0, 10, or 100 μM L-NAME for 2 h before addition of LH (400 ng/ml) or medium control for 6 h, and the effects on AREG, EREG, and PTGS2 mRNA levels were measured by real-time PCR. We also measured the effect on steroidogenic acute regulatory protein (STAR) mRNA levels as a control, as this gene is not acutely regulated by LH in this cell model [22]. The effect of L-NAME on PTGS2 protein levels was assessed by pretreating cells with 0 or

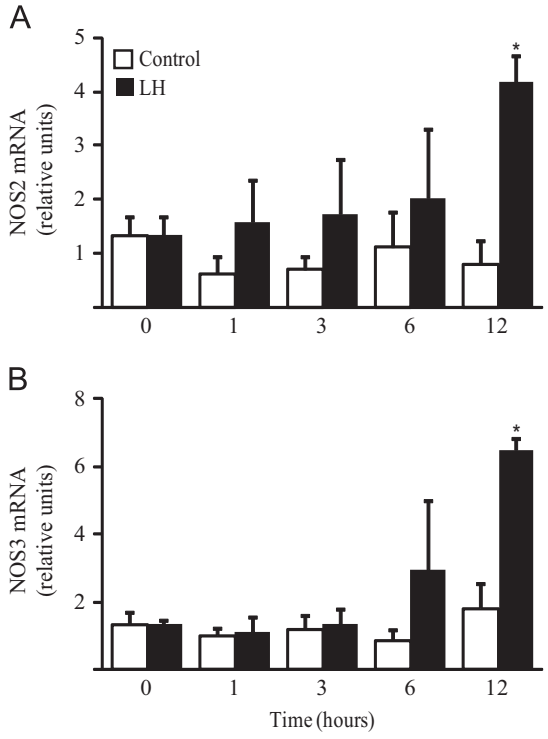

Fig. 1. LH induces NOS mRNA abundance in bovine granulosa cells in a time-dependent manner. Granulosa cells from large follicles (≥ 10 mm diameter) were cultured with serum for 24 h, and then in serum-free medium for a further 18 h, before LH (400 ng/ml) or vehicle control was added for the times given. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM for three independent replicate cultures. LH-stimulated data are expressed relative to the control data at each time point. *$P < 0.05$, significant increase over time-matched control (Dunnett's test).