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Caffeine stimulates *in vitro* pituitary LH secretion in lipopolysaccharide-treated ewes

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ABSTRACT

The study was designed to determine the effects of caffeine on luteinizing hormone (LH) secretion and gene expression of caffeine-associated receptors in anterior pituitary (AP) explants obtained from saline- and lipopolysaccharide (LPS)-treated ewes. Animals had been treated with LPS or saline daily for seven days. Three hours after the last injection of LPS/saline, the AP were collected and divided into four explants. The explants were incubated with: 1/medium-199 (control explants), 2/gonadotropin-releasing hormone (GnRH; 100 pmol/mL; a positive control), 3/caffeine (10 mmol/L), or 4/GnRH + caffeine. Caffeine stimulated ($p < 0.05$) LH release by explants from both saline (19.7 *vs.* control 12.6 ng/mg) and LPS (28.3 *vs.* control 13.9 ng/mg) treated animals. The effect of caffeine on LH secretion was stronger in the LPS-treated group than in saline-treated group, and the observed LH release was similar to that induced by GnRH alone (27.2 ng/mg). Caffeine increased ($p < 0.05$) LHβ gene expression only in explants from LPS-treated animals. In conclusion, the results of the present study demonstrated a stimulatory *in vitro* effect of caffeine on LH secretion by ovine pituitary explants. The potency of the caffeine-induced LH secretion was affected by *in vivo* treatment of the animals with endotoxin.

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is a natural alkaloid found in the leaves, seeds, or fruits of at least 63 plant species worldwide [1]. Natural sources of caffeine, including coffee beans, tea leaves, kola nuts, bissy nuts, cacao beans, guarana, and mate, are used as ingredients to foods, beverages, herbal supplements and medications [2]. The most important mechanism of caffeine action is *via* antagonizing adenosine

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receptors (ADORAs). Adenosine is a locally released purine that may increase or decrease cellular concentrations of cyclic adenosine monophosphate (cAMP). Caffeine selectively blocks adenosine receptors and competitively inhibits the action of adenosine [3]. Caffeine action can also be mediated *via* ryanodine receptors (RyRs). Mammalian tissues express three receptor isoforms, RyR1, RyR2, and RyR3, which are encoded by different genes. RyR1 and RyR2 are expressed predominantly in the sarcoplasmic reticulum of the skeletal muscle and heart, respectively, and they play an essential role in triggering muscle contraction [4,5]. RyR3 was originally identified in the brain, but all three isoforms are expressed in the brain and the major brain isoform is RyR2 [6,7]. RyRs are often co-expressed with inositol 1,4,5-trisphosphate receptors (InsP3R). Caffeine is known as a RyR agonist that induces Ca^{2+} release from intracellular Ca^{2+} stores. However, the precise mechanism through which caffeine activates RyRs remains elusive [8].

Many studies have demonstrated that caffeine is a risk factor for delayed conception [9]. Wilcox et al. [10] reported that women who attempted to become pregnant for three cycles and who were high caffeine consumers were less likely to become pregnant in each cycle compared with lower caffeine consumers. However, some studies failed to identify a correlation between caffeine consumption and delayed conception [11]. Caffeine may affect reproduction processes *via* multiple biological pathways. *In vitro* studies demonstrated direct effects of ADORA1 [12] on pituitary gonadotropes and the presence of RyR2 and RyR3 in αT3-1 gonadotrope cell line [13], which suggests that caffeine may directly affect pituitary secretion of luteinizing hormone (LH).

We showed in our previous study that activation of the immune system may disrupt reproductive processes. The inflammation caused by the peripheral administration of the bacterial endotoxin lipopolysaccharide (LPS) significantly decreased gonadotropin-releasing hormone (GnRH) and LH secretion [14–16]. It was postulated that immune stress affects GnRH/LH secretion at the hypothalamic level largely *via* the action of pro-inflammatory cytokines [17–19]. However, cytokine receptors are also expressed in pituitary cells [20], which enables the direct action of inflammatory mediators on the secretory activity of the anterior pituitary (AP). An *in vivo* study performed on ovariectomized ewes showed that the inflammation induced by bacterial endotoxin affects the pituitary responsiveness to GnRH [21]. Our previous study indicated that reduced responsiveness of the pituitary to GnRH may result from a lower synthesis of GnRH receptors (GnRH-R) during acute inflammation [16]. These findings suggest that the endotoxin treatment may have a profound effect on pituitary function and may determine the sensitivity of the pituitary in response to different factors affecting its secretory activity. Previously, we found that the pituitaries retain a ''memory'' of the events triggered by exposure to LPS. Acute inflammation affected the activity of the pituitary in a prolonged manner and might affect its function even for many hours after the deprivation of inflammatory signals [22]. The current study was designed to determine the effects of caffeine on LH secretion by AP explants and mRNA expression of GnRH-R, ADORAs and RyRs as well as to determine whether a prolonged LPS treatment affects the pituitary response of the ewes to caffeine.

2. Materials and methods

2.1. Animals and experimental design

These studies were performed on adult, three-year-old Merino ewes during the anestrous season (April–May). The animals were maintained indoors in individual pens and were exposed to natural daylight. The ewes were in good condition, *i.e.*, their body condition was estimated as 3 according to a five-point scale [23], and the animals acclimated to the experimental conditions for one month. The ewes were always within visual contact with other members of the flock to prevent isolation stress. The animals were fed a constant diet of commercial concentrates with hay and water available *ad libitum*, according to the recommendations proposed by the National Research Institute of Animal Production for adult ewes [24]. All procedures were performed with the consent of the Local Ethics Committee of Warsaw Agriculture University.

The animals ($n = 12$) were randomly divided into two groups: control ($n = 6$) and LPS-treated ($n = 6$) ewes. The animals were treated with LPS for seven days, and immune stress was induced *via* daily i.v. injection of the appropriate volume of LPS from *E. coli* 055:B5 (400 ng/kg) (Sigma–Aldrich, St. Louis, MO, USA) dissolved in saline (0.9% (w/v) NaCl) (Baxter, Deerfield, IL, USA) into the jugular vein. The maximum volume of LPS solution (10 mg/L) injected into any animal never exceeded 2.5 mL. The control group received an equivalent volume (based on their body weight) of NaCl. The efficiency of the LPS treatment to induce an inflammatory response in the animal was evaluated through the measurement of the animal's body temperature 1 h before and 3 h after the injection.

2.2. Incubation of pituitary explants

All animals were euthanized by decapitation three hours after the LPS or saline injection. The brain was immediately removed from the skulls, and the AP was dissected and divided into four fragments (explants). The explants were first pre-incubated for 1 h in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with medium 199 (M199; 600 μL). The medium was replaced with fresh medium every 15 min. Next, all explants were incubated for additional 30 min in M199. Finally, the explants from each ewe were treated with: 1/M199 only (control explants), 2/GnRH (100 pmol/mL; Sigma–Aldrich) (a positive control), 3/caffeine (10 mmol/L; Sigma–Aldrich), or 4/GnRH + caffeine, and incubated for 3 h at 37 °C (87% O_2, 5% CO_2). The incubation medium consisted of M199 HEPES with Earle's salts, sodium bicarbonate, and HEPES (25 mM) with penicillin–streptomycin (10 mL/L) (Sigma–Aldrich). To avoid the effects of the accumulation of an extensive amount of pituitary hormones in the experimental media, the medium was replaced with fresh medium every 30 min throughout the 3-h period of incubation and then stored at −80 °C until further assay. After incubation, the explants were weighed, immediately frozen in liquid nitrogen and stored at −80 °C until gene expression assay.