**Intracellular Mechanisms of Growth Hormone Action on Apoptosis in Cultured Porcine Ovarian Granulosa Cells**

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**ABSTRACT:** The aims of this study were to detect spontaneously occurring apoptosis in cultured porcine ovarian cells, to examine the role of growth hormone (GH), tyrosine kinase (TK), protein kinase G (PKG) and cyclin-dependent kinase (CDK) in the control of this process, and to determine whether the effect of GH on apoptosis is mediated by TK-, PKG- and cdc2-dependent intracellular mechanisms. We studied the action of pGH (10 ng/ml), blockers of TK (genistein, lavendustin, both 100 ng/ml), PKG (Rp-Br-PET-cGMPS, 50 nM; KT5823, 100 ng/ml) and CDK (olomoucine, 1 µg/ml), as well as combinations of GH with these blockers, on the onset of apoptosis in cultured granulosa cells isolated from antral (3-6 mm) porcine follicles. The functional characteristics of an early apoptotic event, DNA fragmentation, were determined using terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labelling (TUNEL), whilst morphological signs of advanced apoptosis such as pyknosis, chromatin marginalization, shrinkage and fragmentation of the nucleus were detected using routine light microscopy. After culture, some ovarian granulosa cells exhibited DNA fragmentation, which in some cases was associated with morphological apoptosis-related changes (pyknosis, chromatin marginalization, shrinkage and fragmentation of the nucleus). GH significantly reduced the proportion of TUNEL-positive cells. Neither TK nor CDK blockers when given alone, significantly affected the percentage of TUNEL-positive cells although both PKG blockers significantly increased this index. Furthermore, TK and PKG blockers given together with GH, prevented or reversed the inhibitory effect of GH on apoptosis, whilst the CDK blocker olomoucine promoted it. These observations demonstrate apoptosis in porcine ovaries and suggest the involvement of GH, TK, PKG and CDK in the control of this process. They also suggest that the effect of GH on ovarian apoptosis is mediated or regulated by multiple signalling pathways including TK-, PKG- and CDK-dependent intracellular mechanisms. *(Asian-Aust. J. Anim. Sci. 2002, Vol 15, No. 7 : 1045-1050)*

**Key Words:** Growth Hormone (GH), Protein Kinase G Tyrosine Kinase, CDC Kinase, Apoptosis, Ovary

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**INTRODUCTION**

Apoptosis can occur in tissues and organs spontaneously or under the influence of several inducers. Spontaneously occurring or hormone-induced apoptosis plays an important role in the control of ovarian cyclicity, remodelling, follicular development, selection and atresia. Apoptotic cells are characterized by cleavage and fragmentation of DNA, condensation of chromatin, disintegration and pyknosis of nuclei, blebbing and formation of apoptotic bodies in cytoplasm and finally fragmentation of cytoplasm and the whole cell (Kaipia and Hsueh, 1997). Information concerning the hormonal regulation of apoptosis in the ovary is poor. The pattern of hormonal effects seems to be dependent on the stage of the follicular cycle. For example, the anti-apoptotic actions of gonadotropins, cAMP analogues (Hirshfield, 1991) and growth hormone (GH; Eisenhauer et al., 1995; Danilovich et al., 2000) were observed in preovulatory but not in pre-antral (Danilovich et al., 2000) or early antral (Chun et al., 1996) rodent follicles, whilst in rodent luteal cells GH promoted apoptosis (Kiya et al., 1999). Generally, GH is considered as a physiological suppressor of apoptosis in rodent (Eisenhauer et al., 1995; Costoya et al., 1999; Danilovich et al., 2000) and bovine (Sirotkin and Makarevich, 1999) ovaries. In porcine ovarian cells apoptosis was previously observed (Guthrie et al., 1998, 2000; Sirotkin et al., 2000), but the regulatory role of GH have not been described.

The extra- and intracellular mechanisms by which GH prevents ovarian apoptosis are poorly understood. The cAMP/protein kinase A (PKA) is assumed to be one possible mediator because in granulosa cells the activation of the cAMP-dependent pathway promotes cell survival (Hsueh et al., 1994) and blockade of cAMP/PKA prevents the apoptosis-suppressive effect of GH (Sirotkin and Makarevich, 1999).

There is evidence that the GH suppression of apoptosis in rodent follicles is partially mediated by IGF-I, since neutralization of endogenously produced IGF-I reversed the suppressive effect of GH (Eisenhauer et al., 1995). Both GH (Campbell, 1997) and IGF-I (Petley et al., 1999) can affect non-ovarian cells through tyrosine kinase (TK)-dependent intracellular mechanisms, but the role of TK in mediating the GH effect on ovarian cell apoptosis remains unclear. The effect of GH on the survival of chamerst ovaries can be via stimulation of protein kinase B, which is dependent on phosphatidylinositol (P13) kinase activation (Costoya et al., 1999). The role of cyclin-dependent kinases

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(CDK) in mediating the GH effect on ovarian apoptosis has not yet been investigated.

A third potential candidate for mediator of the GH effect is cGMP/protein kinase G (PKG). A pharmacological activator of this mechanism (a nitric oxide donor) was, like GH, able to suppress apoptosis in rodent ovarian cells (Chun et al., 1995), whilst inhibitors of PKG promoted DNA fragmentation in porcine cells (Sirotkin et al., 2000). Direct evidence for the involvement of a cGMP/PKG-dependent signaling pathway in mediating GH effects is not available and the roles of other potential signaling pathways (TK- and CDK-dependent) action remain to be studied.

The aims of our experiments were to identify spontaneously occurring apoptosis in cultured porcine ovarian cells, to examine the role of GH, TK, PKG and CDK in controlling the spontaneous onset of apoptosis and to determine whether the actions of GH on apoptosis are mediated by TK-, PKG- and CDK-dependent intracellular mechanisms. For this purpose, we studied the action of pGH, blockers of TK (genistein, lavendustin), PKG (Rp-Br-PET-cGMPS or KT5823) and CDK (olomoucine), as well as combinations of blockers and GH, on apoptosis in cultured porcine granulosa cells.

MATERIALS AND METHODS

Preparation, culture and processing of granulosa cells

Granulosa cells were obtained from antral follicles, 3-6 mm diameter, from the ovaries of non-cycling Slovakian white gilts (200 days of age), after slaughter at a local abattoir. They were processed and cultured in Lab-Tek chamber-slides (Nunc, Inc., Naperville, USA) as described previously (Sirotkin and Makarevich, 1999). Briefly, cells (1×10^6 cells/ml) were pre-cultured for 7 days in culture medium (DME/F12, Sigma, St.Louis, USA) in the presence of 10% FCS (PAN, Aidenbach, Germany) (200 µl/well) to form a confluent monolayer. The medium was then replaced by fresh medium and culture continued for 2 days in the presence or absence of the following substances: (1) immunological grade porcine GH (pGH; USDA-pGH-B-1, 10 ng/ml, kindly provided by Dr. A. F. Parlow, National Hormone and Pituitary Program, Torrance, USA), (2) inhibitors of TK (genistein and lavendustin, from RBI, Natick, USA, both 100 ng/ml), (3) inhibitors of PKG (Rp-Br-PET-cGMPS from BIOLOG Life Science Institute, Bremen, Germany, 50 nM and KT5823 from Calbiochem-Novabiochem Corp., LaJolla, USA, 100 ng/ml) and (4) an inhibitor of CDK olomoucine (Calbiochem-Novabiochem Corp., 1 µg/ml). These treatment were used either alone or in combination with pGH at the concentrations mentioned above. Previous studies of concentration-dependent effects of GH and protein kinase blockers (Sirotkin and Makarevich, 1999; Sirotkin et al., 2000) showed well-defined effects on ovarian apoptosis at the concentrations listed above. KT5723 and olomoucine were dissolved in 50 µl of DMSO to a final concentration of 1 mg/ml. Immediately before experiment, these stock solutions were dissolved in incubation medium so that the content of DMSO did not exceed 0.01% of medium. Other substances were dissolved directly in medium immediately before experiment. Control groups contained either no cells (blank control) or cells with no added hormone or drugs.

TUNEL assay

At the end of culture the chamber-slides were subjected to the TUNEL (TdT-mediated dUTP nick end labeling) assay as described previously (Sirotkin and Makarevich, 1999) using In Situ Cell Death Detection Kit and DAB reagent (Boehringer Mannheim, GmbH, Mannheim, Germany). Cells containing intensive TdT-positive staining in the nuclei were considered apoptotic. Fixed and permeabilized cells incubated without TdT but with secondary HRP-conjugated antibody and DAB, were used as negative controls. Permeabilized cells incubated with bovine pancreatic DNase I (Boehringer Mannheim GmbH, 0.01 mg/ml; 10 min at room temperature) before TdT treatment to induce DNA fragmentation, were used as positive controls. The general cell morphology and percentage of TUNEL-positive cells in each culture was determined by visual evaluation of cultures and counting of TUNEL-positive and TUNEL-negative cell number using light microscopy.

Statistics

Each experimental group was represented by four chambers. Proportions of TUNEL-positive cells were calculated on the basis of inspection of a minimum 1,000 cells per chamber. The data shown are means of values obtained in three separate experiments performed on separate pools of granulosa cells, each obtained from 20-40 animals. The coefficients of variation between the replicates within each group did not exceed 12%. Significant differences between the experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by the Chi-square test. Differences between the experimental and control groups in the proportions of cells containing TdT immunoreactivity, with p<0.05, were considered significant.

RESULTS

About 10% of granulosa cells in long-term culture showed TUNEL immunoreactivity characteristic of DNA fragmentation. Apoptotic cells exhibited intensive or moderate dark staining of the nuclei (figure 1). Some
TUNEL-positive cells showed morphological signs of apoptosis, such as pyknosis, marginalization of chromatin, shrinkage and fragmentation of the nuclei, resulting in the formation of apoptotic bodies. In contrast, we observed no TUNEL-negative cells with morphological signs of apoptosis (figure 1a and 1b). The negative control cells (TdT omitted) did not show TUNEL-positive staining, although pyknotic nuclei were detected (figure 2).

The proportion of functionally apoptotic (TUNEL-positive) cells was altered by GH and kinase blockers (figure 3-5). Growth hormone treatment reduced the frequency of TUNEL-positive cells by half. Neither of the TK blockers, genistein and lavendustin, given alone affected the percentage of TUNEL-positive cells. On the other hand, they prevented and reversed the inhibitory effect of GH. Genistein caused greater reversal of GH-induced inhibition than lavendustin (figure 3).

PKG blockers, Rp-Br-PET-cGMPS and KT5823 when given alone, significantly stimulated apoptosis in granulosa cells. Moreover, they prevented and reversed the inhibitory effect of GH on this process (figure 4). Olomoucine, a CDK blocker, did not affect the occurrence of apoptosis when given alone but intensified the effect of GH; cells treated with GH and olomoucine showed a significantly lower proportion of TUNEL-positive cells than control cells or cells treated with olomoucine or GH alone (figure 5).

**DISCUSSION**

The signs of apoptosis in ovarian cells observed in the present study are similar to those described previously in rodents (Chun et al., 1996; Eisenhauer et al., 1995), cows (Sirotkin and Makarevich, 1999) and pigs (Guthrie et al., 1998, 2000; Sirotkin et al., 2000). An association of morphological and functional (TUNEL-nicked DNA fragmentation) signs of apoptosis in mammalian ovaries has been described (Kaipia and Hsueh, 1997), but nothing is known about the correlation between these parameters. On the other hand, in non-ovarian reproductive tissues, whilst a cell with fragmented morphology can be TUNEL-negative (Byrne et al., 1999), not all TUNEL-positive nuclei are in the process of programmed cell death (Brison and Schultz, 1997). In our experiments we observed co-incidence of TUNEL-positivity with morphological signs of apoptosis in several cells but an absence of morphological signs in TUNEL-negative cells. This suggests that the TUNEL assay is a reliable method for the detection of the early stages of apoptosis, before any other morphological apoptosis-associated events have appeared. In our study,
only the TUNEL-positive stained cells were considered as apoptotic.

The significant reduction in the proportion of TUNEL-positive cells after GH treatment, observed in our experiments, provides evidence that GH can be a physiological inhibitor of apoptosis in porcine ovaries as it is in rodent (Eisenhauer et al., 1995; Costoya et al., 1999; Danilovich et al., 2000) and bovine (Sirotkin and Makarevich, 1999) ovaries, at least as far as medium-sized antral follicles are concerned. This suggests indirectly that GH controls ovarian follicular development, selection, remodelling, atresia and other apoptosis-associated events.

The observed anti-apoptotic effect of GH can be mediated by IGF-I since (1) GH stimulates ovarian IGF-I release (Sirotkin and Makarevich, 1999); (2) IGF-I, like GH, prevents ovarian apoptosis (Hsueh et al., 1994; Eisenhauer et al., 1995; Kaipia and Hsueh, 1997; Guthrie et al., 1998; Sirotkin and Makarevich, 1999) ovaries, at least as far as medium-sized antral follicles are concerned. This suggests indirectly that GH controls ovarian follicular development, selection, remodelling, atresia and other apoptosis-associated events.

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The stimulatory action of PKG (but not TK or CDK) blockers on the percentage of TUNEL-positive cells, together with the action of cGMP/PKG regulators on apoptosis in ovarian follicular cells reported earlier (Chun et al., 1995; Sirotkin et al., 2000), provides evidence on the involvement of PKG-dependent pathway in down-regulation of ovarian apoptosis. The inhibitory effect of

**Figure 3.** Effect of GH (10 ng/ml medium), TK blockers genistein (G, 100 ng/ml) or lavendustin (L, 100 ng/ml) and combination of GH+G and GH+L (concentrations as above) on the expression of apoptosis in cultured porcine granulosa cells (percentage of TUNEL-positive cells). Values are means±S.E.M., * - Significant difference (p<0.05) compared with control (medium without additions).

**Figure 4.** Effect of GH (10 ng/ml medium), PKG blockers Rp-Br-PET-cGMPS (50 nM) or KT5823 (100 ng/ml) or combination of GH+Rp-Br-PET-cGMPS and GH+KT5823 (concentrations as above) on the expression of apoptosis in cultured porcine granulosa cells (percentage of TUNEL-positive cells). Values are means±S.E.M., * - Significant difference (p<0.05) compared with control (medium without additions).

**Figure 5.** Effect of GH (10 ng/ml medium), cdc2 blocker olomoucine (O, 50 nM) or combination of GH+O (concentrations as above) on the expression of apoptosis in cultured porcine granulosa cells (percentage of TUNEL-positive cells). Values are means±S.E.M., * - Significant difference (p<0.05) compared with control (medium without additions).
PKG blockers on both apoptosis (reported here) and release of IGF-I (Sirotkin et al., 2000), a known anti-apoptotic agent (Hsueh et al., 1994; Eisenhauer et al., 1995; Kaipia and Hsueh, 1997; Guthrie et al., 1998; Sirotkin and Makarevich, 1999); suggest that PKG can promote ovarian cell survival probably not through IGF-I. Furthermore, lack of coincidence of TK blockers effect on porcine ovarian apoptosis (no effect) and IGF-I (stimulatory effect, Makarevich et al., 1997) suggest, that TK-dependent mechanisms of control of apoptosis, similar to PKG-dependent mechanisms, are IGF-I-independent. Interrelationships between CDK and IGF-I in control of ovarian apoptosis remain to be unknown yet. Thus, the role of IGF-I in mediating effect of GH and particular protein kinase-dependent intracellular mechanisms requires further studies.

The ability of PKG, TK and CDK blockers to modify the GH effect on apoptosis in porcine ovarian cells demonstrates that PKG, TK and CDK are involved in the control of cell survival. Furthermore, the prevention and reversal of the effects of GH by PKG and TK inhibitors demonstrates that GH can suppress apoptosis through the activation of TK- and PKG-dependent intracellular mechanisms. The ability of the CDK blocker to modify the anti-apoptotic effect of GH suggests the involvement of CDK in mediating this effect. The fact that the CDK blocker promoted rather than suppressed GH action, indicates that of GH action is down-regulated by CDK. The activation of CDK, in contrast to other kinases, is associated with G1/G2 stages of the cell cycle (Jones and Kazlauskas, 2001), which suggest that this may be the site of GH action.

Some of the effects of kinase blockers described here could be primary while others could be secondary. For example, cGMP/PKG can affect apoptosis through the cAMP/PKA system (Sirotkin et al., 2000), which is known to mediate the action of GH on ovarian apoptosis (Sirotkin and Makarevich, 1999). TK, operating through MAP and PI3 kinases, can stimulate CDK (Jones and Kazlauskas, 2001); this suggests that GH may affect apoptosis through TK and CDK, although the effects of the TK and CDK blockers do not support this hypothesis.

Understanding the fine interrelationships between GH, TK, PKG and CDK requires further studies, including a detailed assessment of the effects of GH and other substances on protein kinases. Nevertheless, this study provides demonstration of apoptosis in porcine ovaries and suggests the involvement of GH, TK, PKG and CDK in its control. It is likely that the effect of GH on ovarian apoptosis is mediated via multiple signaling pathways, including TK-, PKG- and CDK-dependent mechanisms.

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