Corticosterone induced apoptosis of mouse oviduct epithelial cells independent of the TNF-α system

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Abstract. It has been reported in recent studies that restraint stress on pregnant mice during the preimplantation stage elevated corticotrophin-releasing hormone (CRH) and glucocorticoid levels in the serum and oviducts; furthermore, CRH and corticosterone (CORT) impacted preimplantation embryos indirectly by triggering the apoptosis of oviductal epithelial cells (OECs) through activation of the Fas system. However, it remains unclear whether TNF-α signaling is involved in CRH- and/or glucocorticoid-induced apoptosis of OECs. In the present study, it was shown that culture with either CRH or CORT induced significant apoptosis of OECs. The culture of OECs with CRH augmented both FasL expression and TNF-α expression. However, culture with CORT increased FasL, but decreased TNF-α, expression significantly. Although knocking down/knocking out FasL expression in OECs significantly ameliorated the proapoptotic effects of both CRH and CORT, knocking down/knocking out TNF-α expression relieved only the proapoptotic effect of CRH but not that of CORT. Together, our results demonstrated that CRH-induced OEC apoptosis involved both Fas and TNF-α signaling. Conversely, CORT-induced OEC apoptosis involved only the Fas, but not the TNF-α, signaling pathway. The data obtained are crucial for our understanding of the mechanisms by which various categories of stress imposed on pregnant females impair embryo development, as well as for the development of measures to protect the embryo from the adverse effects of stress.

Key words: Apoptosis, Corticotrophin-releasing hormone (CRH), Glucocorticoids, Oviductal epithelial cells, TNF-α signaling (J. Reprod. Dev. 67: 43–51, 2021)
Materials and Methods

Animal care and handling were conducted according to the guidelines approved by the Shandong Agricultural University Animal Care and Use Committee, P. R. China (Permit number: SDAUA-2019-004). Unless otherwise mentioned, all the chemicals and reagents employed in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animals

Mice of the Kunning strain were employed in most experiments conducted for the present study; they were originally derived from ICR (CD-1) mice. The Kunning mice were bred in our laboratory (Shandong Agricultural University). Generalized lymphoproliferative disorder (gld) mice with a germline mutation F273L in FastL in a C57BL/6J genomic background were obtained from the Key Laboratory of Stem Cell Biology, Shanghai Institute of Biological Sciences, China. TNF-α−/− mice with a C57BL/6J genomic background were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China. Wild-type C57BL/6J mice were purchased from Shandong University Center for Laboratory Animals, Jinan, China. All the mice were kept in rooms under a photoperiod of 14-h light/10 h darkness, with lights off at 2000 h.

Culture of oviduct epithelium cells (OECs)

Female mice (8–12 weeks old) were injected with eCG (10 IU/ mouse); at 48 h after eCG injection, the mice were sacrificed to recover oviducts. The oviducts recovered from three mice were cut into segments, and each segment was squeezed using forceps to release the oviduct epithelium tissue. The tissue blocks obtained were digested with 0.25% trypsin at 37°C for 30 min and washed twice by centrifugation (200 g, 5 min). The pellets were resuspended in 2 ml of Dulbecco’s modified Eagle’s medium/Ham’s F12 (DMEM/F12; Gibco, Beijing, China) supplemented with 10% fetal calf serum (Gibco) and 0.5% penicillin-streptomycin solution (Gibco). The final suspension was added to two wells (1 ml per well) of a 12-well culture plate and cultured at 37.5°C under a humidified atmosphere of 5% CO2 in air. When they grew to about 80–90% of confluence at 18–24 h of culture, the cells in each well were digested with trypsin, resuspended in 2 ml DMEM/F12, and cultured in two wells (1 ml per well) of a 12-well culture plate. When the subcultures reached approximately 50% confluence at 24–36 h of culture, the OECs were cultured for 48 h in the presence of CRH or CORT before further analysis. The concentrations of CRH and CORT used were 2 × 10−6 M and 1 × 10−5 M, respectively, which can induce apoptosis of OECs both efficiently and reversibly [16]. To prepare stock solutions, CRH and 1 × 10−6 M CORT (10 mM) were dissolved in water, and CORT (10 mM) was dissolved in ethanol. The stock solutions were stored at −20°C until use.

Apoptosis assessment of OECs by JC-1 staining

Cultured OECs were stained in situ in wells of a 12-well plate using 0.01 mg/ml of Hoechst 33342 for 5 min in the dark. The stained cells were examined under a Leica DMLB fluorescence microscope at a magnification of 400×. Six fields were randomly observed in each well. The heterochromatin was heavily stained and was identified by its characteristic bright fluorescence. While the apoptotic cells showed pyknotic nuclei full of heterochromatin, healthy cells showed normal nuclei with sparse heterochromatin spots. To reduce subjectivity, percentages of the apoptotic cells were always calculated in a double-blind approach by two investigators.

Apoptosis assessment of OECs by flow cytometry

Annexin/PI staining was performed using a BD Pharmingen FITC Annexin V Apoptosis Detection Kit (BD Biosciences, 0027279). Briefly, the spent medium was collected, to the maximum possible extent, from wells with cultured OECs and preserved for future use. After the OECs were washed twice with cooled PBS, 200 µl PBS containing 0.25% trypsin was added to each well. Digestion was carried out at 37.5°C for 3 to 4 min, and terminated by adding the spent medium collected previously. The cells were then dispersed by repeatedly pipetting in PBS, and the resultant suspension was centrifuged at 200 × g for 5 min. After the supernatant was removed, the cells were resuspended in cooled PBS and washed twice by centrifugation at 200 g for 5 min. Thereafter, cells were resuspended in 100 µl of 1 × staining buffer, 5 µl Annexin V-FITC, and 5 µl PI staining solution, and stained for 15 min at 37°C in the dark. At the end of staining, 400 µl of 1 × staining buffer was added and the mixture was cooled on ice. Flow cytometry was performed on the stained cells using BD LSR FortessaTM within 1 h after staining to assess apoptosis. We analyzed the data using FlowJo software (FlowJo 10 LLC, TreeStar, Ashland, OR, USA). The cell populations were gated according to the gating information of non-dyed control cells and annexin V- or PI-dyed control cells. The lower left quadrant contains the healthy (double-negative) population, the lower right quadrant contains the early apoptotic (annexin V-positive/PI-negative) population, the upper left quadrant contains the damaged (annexin V-negative/PI-positive) cells, and the upper right quadrant contains the late apoptotic and necrotic (double-positive) cells.

Real-time PCR

To isolate RNA, the spent medium was removed to the maximum possible extent from wells with cultured OECs, and 1 ml Trizol reagent was added to each well. The cells were pipetted for 5 min to facilitate lysis. Thereafter, RNA was resuspended in DEPC-dH2O before digestion with RNase-free DNase 1 (Takara Biotechniques, Dalian, China). The RNA obtained was spectroscopically quantified at 260 nm. The ratio of A260:A280 (1.8–2.0) was determined and electrophoresis in 1% agarose was carried out to assess RNA purity and integrity.

Reverse transcription was conducted using a total volume of 20 µl and Superscript III Reverse Transcriptase (Invitrogen Australia Pty., Mulgrave, Australia). First, 2 µl of RNA sample, 1 µl Oligo dT18 (Takara), and 10 µl DEPC-dH2O were mixed in a reaction tube, and the mixture was incubated at 65°C for 10 min in a PCR instrument (Thermo Scientific, Hudson, NH, USA). Second, the reaction tube was cooled for 2 min on ice before a brief centrifugation (200 × g, 4°C) step. Third, 5× reverse transcription buffer (4 µl), RNase inhibitor (0.5 µl), dNTP (2 µl), and Superscript III Reverse Transcriptase (0.5 µl) were added to the reaction tube. The mixture was incubated at 42°C for 1 h, at 70°C for 15 min, and stored at −80°C until use.

Gene-specific primers used are as follows: For Bcl2, forward 5′- TTC GGG ATG GAG TAA ACT GG -3′, reverse 5′- TGG ATC...
Corticosterone-induced apoptosis in oviduct epithelial cells

Effects of in vitro exposure to CRH or CORT on apoptosis of OECs

When mouse OECs harvested 48 h post eCG injection attained 70–90% of confluence (Fig. 1A), they were sub-cultured. When the subcultures reached approximately 50% confluence (Fig. 1B), the OECs were cultured for 48 h in the presence of 2 × 10⁻⁶ M CRH or 1 × 10⁻⁵ M CORT before the assessment of apoptosis. Control OECs were cultured for 48 h without CRH or CORT. Hoechst staining showed that the percentage of apoptotic cells was significantly higher in OECs cultured with CRH/CORT than in control OECs (Figs. 1C, D, E, and F). Our flow cytometry results showed that while the percentage of cells undergoing early or late apoptosis/necrosis was significantly higher (Figs. 2A to D), the ratio of Bcl2/Bax mRNAs was significantly lower (Fig. 2E) in OECs cultured with than without CRH or CORT. Consequently, it was inferred that the culture of OECs with either CRH or CORT induced severe apoptosis.

Effects of in vitro exposure of OECs to CRH or CORT on the FasL and TNF-α expression

The OECs were cultured for 48 h in the presence of CRH or CORT before ELISA-based measurement of FasL and TNF-α levels. Culture with either CRH or CORT significantly enhanced FasL expression (Fig. 3A). However, while culture with CRH augmented TNF-α expression, culture with CORT significantly diminished TNF-α expression (Fig. 3B). Thus, while CRH triggered OEC apoptosis through both the Fas system and TNF-α signaling, CORT did so through Fas signaling but not through TNF-α signaling.

Effects of knocking down FasL or TNF-α on CRH- or CORT-induced apoptosis of OECs

To evaluate the regulatory role of FasL and TNF-α in CRH- or CORT-induced apoptosis of OECs, FasL and TNF-α were knocked down by RNA interference. To assess the efficiency of different siRNA sequences, FasL and TNF-α levels were measured by ELISA in cells transfected with FasL siRNA and TNF-α siRNA, respectively. Levels of FasL and TNF-α declined significantly following transfection with FasL siRNA-2 and TNF-α siRNA-2 and -3 compared to the corresponding levels following transfection with negative control siRNA (Figs. 4A and B). Thus, FasL siRNA-2 and TNF-α siRNA-2 were used in the subsequent experiments.

When cultured in the presence of CRH, the percentage of cells undergoing early or late apoptosis/necrosis was significantly lower (Figs. 4C to F) and the ratio of Bcl2/Bax mRNAs was significantly higher (Fig. 4G) in OECs transfected with either FasL siRNA-2 or TNF-α siRNA-2 than in those transfected with negative control siRNA. The results indicated that knocking down either FasL or TNF-α significantly ameliorated the proapoptotic effects of CRH on OECs. When cultured in the presence of CORT, however, only transfection with FasL siRNA-2 decreased the percentage of cells undergoing early and late apoptosis/necrosis (E + L/N) significantly compared to that following transfection with negative control siRNA. However, transfection with TNF-α siRNA-2 did not impact the percentages of E + L/N cells (Figs. 4H, J, K, and L). While transfection with FasL siRNA-2 increased the ratio of Bcl2/Bax mRNAs,
transfection with TNF-α siRNA-2 decreased the ratio significantly (Fig. 4I). Taken together, the results indicated that CRH-induced OEC apoptosis involved both Fas signaling and TNF-α signaling; conversely, CORT-induced OEC apoptosis involved only Fas signaling but not TNF-α signaling.

Effects of knocking out FasL or TNF-α on CRH- or CORT-induced apoptosis of OECs

To further verify the role of FasL and TNF-α in mediating the CRH- or CORT-induced apoptosis of OECs, OECs recovered from the gld mice harboring a FasL mutation and those from the TNF-α knockout (TNF-α–/–) mice were cultured for 48 h in the presence of CRH or CORT before assessment for apoptosis by flow cytometry. OECs recovered from C57BL/6J mice were also cultured with CRH or CORT for the same period to serve as controls. When cultured with CRH, while the percentages of healthy cells were higher, those of early and late apoptotic/necrotic cells were significantly lower in both gld and TNF-α–/– OECs than in C57 OECs (Figs. 5A, C, D, and E). However, when cultured with CORT, while the percentages of early and late apoptotic/necrotic cells were lower in gld OECs than in the control C57 OECs, these percentages did not differ between the TNF-α–/– OECs and the control C57 OECs (Figs. 5B, F, G, and H). Taken together, the results further confirmed that while CRH induced apoptosis in OECs by activating both Fas signaling and TNF-α signaling, CORT triggered OEC apoptosis by activating the Fas system but not involving TNF-α signaling.

Discussion

The findings of the present study demonstrated that culture with either CRH or CORT induced significant apoptosis of OECs. While treatment of OECs with CRH augmented both FasL expression and TNF-α expression, treatment with CORT increased FasL expression while concomitantly diminishing TNF-α expression. Although knocking down or knocking out FasL expression in OECs significantly ameliorated the proapoptotic effects of both CRH and CORT, knocking down or knocking out TNF-α expression relieved only the proapoptotic effect of CRH but not that of CORT. In summary, the results indicated that while CRH induced OEC apoptosis through both Fas signaling and TNF-α signaling, CORT induced OEC apoptosis only via Fas, without the involvement of TNF-α signaling.

Prior studies have reported that CRH triggers apoptosis in different cells in vitro. For instance, Zhang and colleagues [18] observed that CRH induced the apoptosis of hippocampal neurons in vitro, and its receptor (CRHR1) is involved in this process. Dermitzaki and colleagues [19] demonstrated that CRH triggered apoptosis in the PC12 rat pheochromocytoma cell line by activating p38 mitogen-activated protein kinase. Ock et al. [20] reported that CRH induced classical apoptosis in mouse microglia cultured in vitro. Jin et al. [21] showed that CRH promoted apoptosis in prostate cancer cells in vitro. Furthermore, treatment of cultured mouse mural granulosa cells (MGCs) with CRH induced significant apoptosis [22]. Glucocorticoids also trigger apoptosis in various cells in vitro.
example, Schmidt and colleagues [23] found that in vitro treatment of human monocytes with glucocorticoids resulted in apoptosis in a time- and dose-dependent manner. Treatment with dexamethasone triggered apoptosis in MLO-Y4 osteocytes [24]. Culture of human fetal ovaries with dexamethasone significantly accelerated the apoptotic rates of germ cells [25]. Furthermore, culture of MGCs with corticosterone induced apoptosis [26].

Minas and colleagues [27] observed that CRH augmented FasL expression in ovarian cancer cells of OvCa3 and A2780 lines through CRHR1. Taliouri and colleagues [28] found that CRH could induce both transcription and translation of FasL in HeLa cells. Petsas and colleagues [29] reported that CRH induced FasL expression in human macrophages and enabled them to induce apoptosis of cocultured Fas-expressing cells. Li and colleagues [22] demonstrated that CRH significantly elevated the levels of Fasl and Fas expression during culture of MGCs. Furthermore, Tan and colleagues [16] showed that CRH augmented the FasL and Fas expression in cultured OECs. However, there are relatively few studies reporting that CRH promotes TNF-α expression in different cells. Song and colleagues [30] reported that CRH facilitated TNF-α production in CD14+ cells and triggered apoptosis in endothelial cells.

**Fig. 2.** Effects of in vitro exposure to corticotrophin-releasing hormone (CRH) or corticosterone (CORT) on the apoptosis of oviductal epithelial cells (OECs). A to C show flow cytometry graphs following annexin-V and PI staining of OECs cultured without (A), or with CRH (B), or CORT (C). Graph D shows percentages of healthy (H), early apoptotic (E), or late apoptotic and necrotic (LA/N) cells, as revealed by flow cytometry. * indicates significant differences (P < 0.05) between two groups. Graph E shows the ratio of Bcl2/Bax mRNAs as measured by RT-PCR in OECs in the Ctrl, CRH, and CORT groups. The ratio in the Ctrl group was set to 1 (dotted line), and the ratio in the treatment groups were expressed relative to it. * indicates significant difference (P < 0.05) from the control group. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice.

**Fig. 3.** Effects of in vitro exposure to corticotrophin-releasing hormone (CRH) or corticosterone (CORT) on FasL and TNF-α expression in oviductal epithelial cells (OECs). Graphs A and B show FasL expression and TNF-α expression (ELISA results), respectively, in OECs after culture with CRH or CORT for 48 h. Control (Ctrl) cells were cultured for 48 h with neither CRH nor CORT. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. * indicates significant differences (P < 0.05) between two groups.
Fig. 4. Effects of knocking down FasL or TNF-α on corticotrophin-releasing hormone (CRH)- or corticosterone (CORT)-induced apoptosis of oviductal epithelial cells (OECs). OECs recovered from the generalized lymphoproliferative disorder (gld) mice with a FasL mutation, the TNF-α knockout (TN–/–) mice, and the control C57BL/6J (C57) mice were cultured for 48 h with CRH or CORT before apoptosis assessment by flow cytometry. Graphs A and B show percentages of healthy (H), early apoptotic (E), or late apoptotic/necrotic (LA/N) OECs following culture with CRH and CORT, respectively. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. * indicates significant differences (P < 0.05) between two groups. Panels C to H show flow cytometry graphs following annexin-V and PI staining after OECs recovered from C57, gld, and TN–/– mice were cultured with CRH (panels C, D and E) or CORT (panels F, G and H).

Fig. 5. Effects of knocking out FasL or TNF-α on corticotrophin-releasing hormone (CRH)- or corticosterone (CORT)-induced apoptosis of oviductal epithelial cells (OECs). OECs recovered from the generalized lymphoproliferative disorder (gld) mice with a FasL mutation, the TNF-α knockout (TN–/–) mice, and the control C57BL/6J (C57) mice were cultured for 48 h with CRH or CORT before apoptosis assessment by flow cytometry. Graphs A and B show percentages of healthy (H), early apoptotic (E), or late apoptotic/necrotic (LA/N) OECs following culture with CRH and CORT, respectively. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. * indicates significant differences (P < 0.05) between two groups. Panels C to H show flow cytometry graphs following annexin-V and PI staining after OECs recovered from C57, gld, and TN–/– mice were cultured with CRH (panels C, D and E) or CORT (panels F, G and H).
[31] demonstrated that CRH enhanced TNF-α expression in ovarian cells. Thus, the present study provides evidence for the first time that CRH induces apoptosis of OECs by activating TNF-α signaling. Treatment of mice with glucocorticoids significantly enhanced FasL expression in testicular germ cells [32], MGCs, and cumulus cells [33]. Culture with glucocorticoids activates the Fas/FasL system in osteocytes [24] and monocytes [34]. Furthermore, culture of mouse OECs with CORT significantly elevated Fas and FasL expression [16]. However, conflicting results have been reported on the effects of CORT on TNF-α expression in testicular germ cells [32], MGCs, and cumulus cells. Thus, the present study provides evidence for the first time that glucocorticoids and CRH induce apoptosis of OECs by activating FasL expression and TNF-α signaling. The data obtained are crucial for our understanding of the mechanisms by which various categories of stress imposed on pregnant females impair embryo development, as well as for the development of measures to protect the embryo from the adverse effects of stress.

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