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Apoptosis- and Endoplasmic Reticulum Stress-Related Genes Were Regulated by Estrogen and Progesterone in the Uteri of Calbindin-D_{9k} and -D_{28k} Knockout Mice

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ABSTRACT
Calcium (Ca^{2+}) is an important regulator of apoptotic signaling. Calbindin-D_{9k} (CaBP-9k) and -D_{28k} (CaBP-28k) have a high affinity for Ca^{2+} ions. Uterine calbindins appear to be involved in the regulation of myometrial activity by intracellular Ca^{2+}. In addition, uterine calbindins are expressed in the mouse endometrium and are regulated by steroid hormones during implantation and development. The aim of the present study was to evaluate the regulation of apoptosis in the uteri of CaBP-9k, CaBP-28k, and CaBP-9k/28k knockout (KO) mice. Our findings indicated that Bax protein was enhanced in the uteri of CaBP-28k and CaBP-9k/28k KO mice compared to wild-type (WT) and CaBP-9k KO mice, but no difference was observed in Bcl-2 protein expression. The expressions of caspase 3, 6, and 7 proteins were higher in both CaBP-28k and CaBP-9k/28k KO mice than in WT and CaBP-9k KO mice. These results suggest that the absence of CaBP-28k increases apoptotic signaling. We also investigated the expression of endoplasmic reticulum (ER) stress genes by Western blot analysis in calbindin KO mice. C/EBP homologous protein and immunoglobulin heavy chain-binding protein protein levels were elevated in CaBP-28k KO mice compared to WT mice. When immature mice were treated with 17β-estradiol (E2) or progesterone (P4) for 3 days, we found that the expressions of Bax and caspase 3 protein were increased by E2 treatment in WT and CaBP-9k KO mice, and by P4 treatment in CaBP-28k KO mice. These results indicate that CaBP-28k blocks the up-regulation of apoptosis-related genes and ER stress genes, implying that CaBP-28k may decrease the expression of genes involved in apoptosis and ER stress in murine uterine tissue. J. Cell. Biochem. 113: 194–203, 2012.
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KEY WORDS: APOPTOSIS; ENDOPLASMIC RETICULUM STRESS; CALBINDIN-D_{9K}; CALBINDIN-D_{28K}

Calcium ions regulate many cellular functions including cell proliferation, differentiation [Richter and Kass, 1991; Dolmetsch et al., 1997]. Calcium ions play a critical role in apoptosis, and increased intracellular calcium has been shown to activate apoptotic pathways [Martikainen et al., 1991; Lynch et al., 2000]. In addition, it has been shown that an elevation or reduction in intracellular calcium ion levels can promote cell death through necrosis or apoptosis [Choi, 1995; Nicotera and Orrenius, 1998]. Increased intracellular calcium levels trigger the release of cytochrome *c* and the activation of caspase 3, leading to cell death [Rizzuto et al., 1998; Csordas et al., 1999].

Mitochondrion-mediated apoptotic plays a central role in animal development and tissue homeostasis is regulated by Bcl-2 family proteins. Apoptosis is regulated by several proteins, including members of the caspase protein family. Bcl-2 is one of a group of anti-apoptotic proteins that can prevent or reduce cell death induced by a variety of stimuli. Bax is a member of the Bcl-2 family and functions as a pro-apoptotic protein in the cytosol. Bax forms a dimer, and thus the increased expression of Bax promotes dimerization and cell death, or Bax can heterodimerize with Bcl-2 to neutralize the anti-apoptotic function of Bcl-2. Bax is essential for the release of cytochrome *c* and caspase activation [Eskes et al., 1998; Finucane et al., 1999; Gross et al., 1999]. Cytochrome *c* binds to Apaf-1 and caspase 9, resulting in the activation of caspase 9, which in turn activates caspase 3 and caspase 7, ultimately resulting in cell death. A recent study demonstrated that Bax can also localize to the endoplasmic reticulum (ER) and is activated in response to ER stress, leading to calcium depletion and murine caspase 12 activation [Scorrano et al., 2003; Zong et al., 2003]. ER stress can also trigger apoptosis.

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Apoptosis signals are generated through several mechanisms, including the induction of C/EBP homologous protein (CHOP) or immunoglobulin heavy chain-binding protein (BiP), an ER chaperone with sentinel activity [Hendershot, 2004; Oyadomari and Mori, 2004; Li et al., 2006].

Calcium-binding proteins (calbindins) are a group of intracellular proteins with a high affinity for calcium and include calmodulin, parvabumin, troponin C, calbindin-D_{9k} (CaBP-9k), and calbindin-D_{28k} (CaBP-28k). CaBP-9k and CaBP-28k are expressed in a variety of organs and tissues, including the intestine, kidney, pancreas, brain, bone, uterus, and placenta [Nys et al., 1992; Mutema and Rhoten, 1994; Bellido et al., 2000; Rabinovitch et al., 2001; Kutuzova et al., 2006]. CaBP-9k and CaBP-28k are involved in intestinal calcium absorption, and are typically regulated at the transcriptional and post-transcriptional level by 1,25-dihydroxyvitamin D3, the hormonal form of vitamin D [Wasserman and Fullmer, 1989; Darwish and DeLuca, 1992]. However, in the uterus, CaBP-9k and CaBP-28k are not regulated by vitamin D, despite the presence of vitamin D receptors in this tissue. Instead, uterine CaBP-9k and CaBP-28k are regulated by sex steroid hormones in rats [L'Horset et al., 1990, 1993]. In a previous report, P4 was shown to enhance CaBP-9k expression in the uteri of oophorectomized adult mice, whereas E2 had no effect [Tatsumi et al., 1999; An et al., 2004a]. In immature mice, it was shown that E2 induced the down-regulation of uterine CaBP-28k expression [Opperman et al., 1992]. CaBP-9k is expressed at high levels in the mouse uterus in diestrus and metoestrus, and at basal levels in proestrus and estrus [Nie et al., 2000; An et al., 2004a]. CaBP-28k is expressed at high levels in human uteri in the mid-secretory phase. It has also been shown that the levels of mouse uterine CaBP-9k and CaBP-28k are increased during early pregnancy and implantation [Tatsumi et al., 1999; Nie et al., 2000; Luu et al., 2004]. These data indicate that CaBP-9k and CaBP-28k are regulated by sex hormones in uterine epithelium and seem to be important factors for implantation in human and mouse.

Apoptosis is a genetically regulated cellular suicide mechanism essential for the removal of damaged or unwanted cells and for the maintenance of tissue homeostasis in multicellular organisms. Recent studies have investigated the role of cell death during development and have revealed that apoptosis is initiated in response to implantation in the uterus [Spencer et al., 1996; Vinatier et al., 1996; Gosden and Spears, 1997; Jacobson et al., 1997; Milligan and Schwartz, 1997]. The regulation of apoptosis by sex steroid hormones is one of the most important functions of 17β-estradiol (E2) and progesterone (P4) in the uterus. E2 has been shown to induce apoptosis in the uterine epithelium, while P4 inhibits this process [Martin et al., 1970; Terada et al., 1989]. However, the detailed mechanisms of regulation of apoptosis in the uterus remain to be elucidated and are essential for understanding regulation of calbindins in apoptosis-induced signaling pathways.

CaBP-28k has been shown to block multiple pro-apoptotic pathways. Expression of CaBP-28k in neural cells inhibited the pro-apoptotic activity of mutant presenilin-1 by preventing calcium-mediated mitochondrial damage and cytochrome *c* release [Guo et al., 1998]. Expression of CaBP-28k in HEK renal cells was shown to inhibit parathyroid hormone-induced apoptosis by buffering intracellular calcium [Turner et al., 2000; Rintoul et al., 2001]. In addition, cytokine-induced apoptosis and necrosis of pancreatic beta cells can be prevented by CaBP-28k [Rabinovitch et al., 2001]. CaBP-28k protects osteoblasts against TNF and glucocorticoid-induced apoptosis through the inhibition of caspase 3 activity [Bellido et al., 2000; Liu et al., 2004]. In addition, the induction of CaBP-9k expression by melatonin was shown to reduce H_2O_2-mediated cell death in rat pituitary GH3 cells [Yoo and Jeung, 2009, 2010]. In previous studies, hydrogen peroxide (H_2O_2)-induced apoptosis can be prevented by CaBP-28k expression in human endometrial Ishikawa cells [Jung et al., 2011].

Although several studies have examined the function of CaBP-9k and CaBP-28k in apoptosis-induced signaling pathways in the uterus, no evidence of transcriptional dysregulation has been previously observed in the CaBP-9k, CaBP-28k, and CaBP-9k/28k knockout (KO) mouse models. Thus, we investigated whether apoptosis-related gene expression is altered by the absence of calbindin genes in these KO mice to better understand the mechanisms of calbindin activity in the uterus.

MATERIALS AND METHODS

ANIMALS

CaBP-9k and CaBP-28k single KO mice were generated as previously described [Kutuzova et al., 2006; Lee et al., 2007]. CaBP-9/28k mice were generated by breeding CaBP-9k single KO female mice with CaBP-28k single KO male mice to generate double heterozygotes, which were subsequently bred to obtain homozygous CaBP-9/28k mice. The genotypes of offspring were determined by PCR analysis, as described previously [Lee et al., 2007]. All experimental procedures and animal use were approved by the Ethics Committee of the Chungbuk National University.

EXPERIMENTAL TREATMENTS

Immature female CaBP-9k KO, CaBP-28k KO, CaBP-9/28k KO, and wild-type (WT) (C57BL/6) mice (7 and 14 days of age) were produced by the breeding of the corresponding heterozygous mice. All animals were housed in polycarbonate cages and were allowed to acclimate to housing in an environmentally controlled room (temperature: $23 \pm 2°C$, relative humidity: $50 \pm 10\%$, frequent ventilation, and a 12-h light cycle). Animals were fed soy-free food pellets (Dyets, Inc., Bethlehem, PA). Only female mice ($n = 20$ total; $n = 5$ per group) were used in this study, and each group was subcutaneously (sc) injection with 10% ethanol vehicle, E2 (40 μg/kg body weight [BW]), or P4 (4 mg/kg BW) for 3 days. The uteri tissues were rapidly excised from euthanized mice for protein isolation. Other uteri were fixed in 10% neutral-buffered formalin solution and sectioned at 5 μm for TUNEL assay.

TUNEL STAINING

TUNEL staining was performed on paraffin-embedded sections using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany). Uteri were embedded in paraffin and then the slides were dewaxed and rehydrated by heating at 60°C. Slides were then washed in xylene and rehydrated through a graded series of ethanol and double-distilled water steps. Next, the slides were incubated for 30 min at 37°C in a 20 μg/ml proteinase K

working solution. The slides were rinsed with PBS, and the area around the sample was dried. The slides were then incubated with 200 µl of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) for 60 min in a dark, humidified atmosphere at 37°C. After slides were rinsed three times with PBS, they were analyzed using a fluorescence microscope (BX51 Standard Microscope, Olympus, Japan). Positive control sections were treated with the same reagents but also pre-treated with 1,000 U/ml DNase I (TaKaRa Bio Inc., Otsu, Shiga, Japan) in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mg/ml BSA for 10 min at room temperature prior to the TUNEL assay. For negative controls, sections were incubated with TUNEL label only. The all slides were then stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St Louis, CA) and viewed with a fluorescence microscope. The numbers of TUNEL-positive cells were counted using fluorescence microscopy.

WESTERN BLOT ANALYSIS

Protein was extracted using Proprep (iNtRON Bio., Inc., Sungnam, Kyungki-do, Korea) according to the supplier's instructions. Protein concentration was determined using the BCA assay (Sigma). Proteins (50 µg) were separated by 12.5% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with antibodies against the following proteins: bax, CaBP-28k (1:1,000) (Santa Cruz Biotech, Santa Cruz, CA), caspase 3, caspase 6, caspase 7 (1:500), bcl-2, CHOP, BiP, CaBP-9k (1:1,000) (Cell Signaling Technology, Beverly, MA), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1,000, Santa Cruz Biotech). Immunoreactive proteins were visualized by exposure to X-ray film. Protein bands were quantified by image scanning, and optical density was measured using a Gel Doc EQ system (Bio-Rad Laboratories, Inc.) after data were corrected by background subtraction and normalized using GAPDH as an internal control.

STATISTICAL ANALYSIS

Significant differences were determined by ANOVA, followed by Tukey's test in experimental groups. Statistical analysis was performed using Prism Graph Pad (v4.0; GraphPad Software Inc., San Diego, CA). The data were presented as mean ± standard error of the mean (SEM) in triplicates derived from three individual experiments. Data were considered statistically significant at $P < 0.05$.

RESULTS

UTERINE APOPTOSIS

A TUNEL assay detected apoptotic cells in the endometrial and myometrium layers of CaBP-9k KO, CaBP-28k KO, CaBP-9/28k KO, and WT uteri. The incidence of apoptosis in the uteri of CaBP-9k KO, CaBP-28k KO, and CaBP-9/28k KO mice was compared with that of WT mice (Fig. 1A). Mouse uterine tissue incubated only with labeling solution was used as a negative control and showed a complete absence of staining. The Apoptotic signals were present in the epithelial cells lining the lumen and myometrium as shown in Figure 1A. CaBP-28k KO and CaBP-9/28k KO mice showed a significant increase in the number of TUNEL-positive cells as seen in Figure 1B. However, the number of TUNEL-positive cells in the uteri of CaBP-9k KO mice was similar to that of WT mice at 7 days after birth.

INDUCTION OF APOPTOTIC SIGNALING IN KO MICE

We compared the expression of apoptosis-related genes in the uterine tissue of immature WT, CaBP-9k KO, CaBP-28k KO, and CaBP-9/28k KO mice. To avoid the fluctuations in gene expression that accompanies estrous cycle in mature mice, only immature mice were utilized. Uterine proteins were isolated from immature CaBP-9k KO, CaBP-28k KO, CaBP-9/28k KO, and WT mice 7 days after birth. We measured the expression of the apoptotic proteins Bax and Bcl-2 in WT, CaBP-9k KO, CaBP-28k KO, and CaBP-9/28k KO mice. Although CaBP-9k protein was not detected in the uteri of immature mice by Western blot analysis in our previous study [Ji et al., 2006], in this study, CaBP-28k protein was detected in the uterine tissue of WT and CaBP-9k KO mice. We also found that apoptosis-related genes were differentially regulated: CaBP-28k KO and CaBP-9/28k KO mice showed significantly higher Bax protein expression compared to WT mice (Fig. 2). However, no difference was observed in Bcl-2, CaBP-9k, or CaBP-28k protein expression among the various mouse groups. In addition, significantly higher levels of activated caspase 3, 6, and 7 proteins were detected in the uteri of CaBP-28k KO and CaBP-9/28k KO mice (Fig. 3), while significantly activated expression of caspase 3 protein was not observed in CaBP-9k KO mice compared to WT mice. These results indicate that the absence of CaBP-28k is associated with increased apoptotic signaling.

ENDOPLASMIC RETICULUM STRESS IN KO MICE

We further compared the expression of the ER stress genes CHOP and BiP in uterine tissue from immature WT, CaBP-9k KO, CaBP-28k KO, and CaBP-9/28k KO mice 7 days after birth. CaBP-28k KO and CaBP-9/28k KO mice showed significantly higher expressions of CHOP and Bip as seen in Figure 4. However, the expressions of CHOP and Bip protein were not altered in CaBP-9k KO mice, suggesting that the absence of CaBP-28k may be associated with increased ER stress.

EFFECTS OF STEROID HORMONES ON APOPTOSIS-RELATED GENES

To confirm the effects of sex steroid hormones on apoptosis-related gene expression, immature WT mice (14 days of age) were given daily injections of E2 (40 µg/kg) or P4 (4 mg/kg) for 3 days. In addition, CaBP-9k KO mice were given injections of E2, and CaBP-28k KO mice were given injections of P4 for 3 days. As seen in Figure 5, E2 decreased CaBP-28k expression in WT and CaBP-9k KO mice, and P4 increased CaBP-9k expression in WT and CaBP-28k KO mice. In addition, E2 treatment up-regulated Bax and caspase-3 protein in WT and CaBP-9k KO mice, and P4 treatment had the same effect in CaBP-28k KO mice. However, the expression of Bcl-2 was not affected by E2 or P4 treatment. These results indicate that CaBP-28k may decrease apoptosis-related gene expression in mouse uterine tissue.

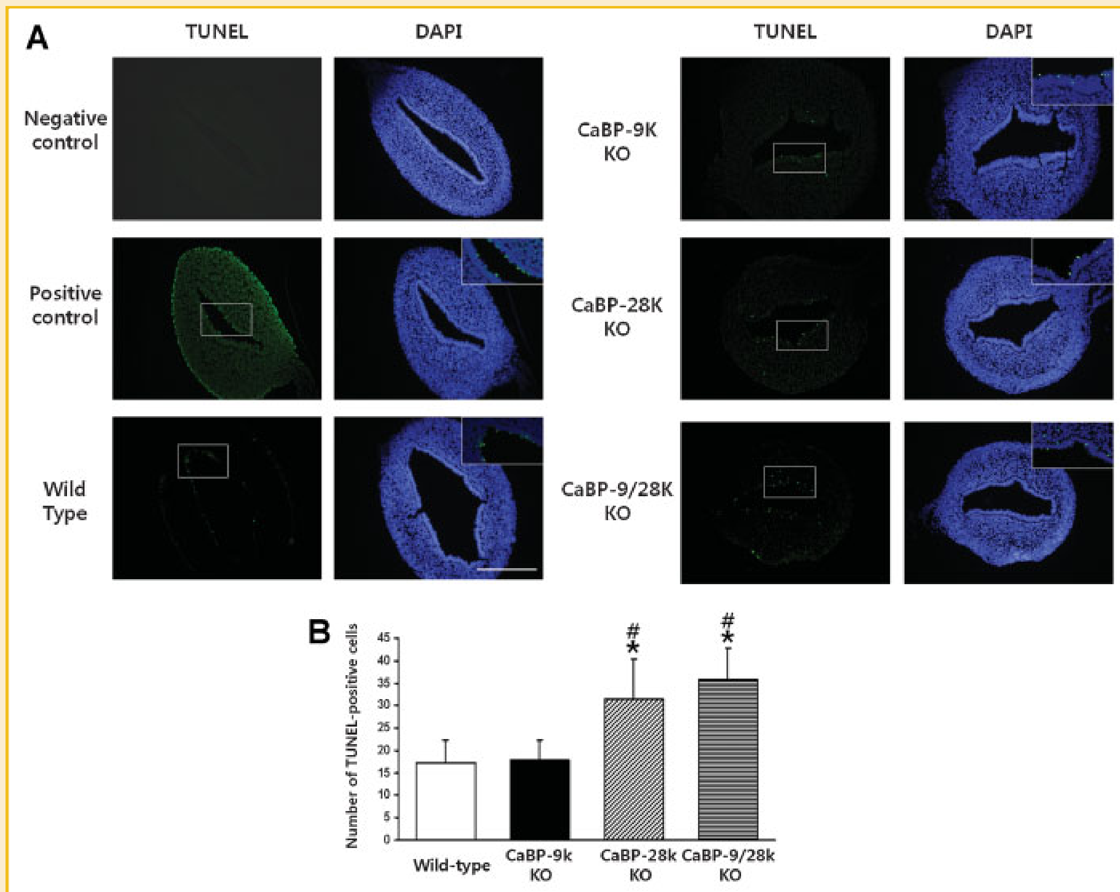

Fig. 1. Apoptosis was evaluated in the uteri of calbindin KO mice by TUNEL assay. Apoptotic nuclei were labeled with TUNEL (green) and DAPI (blue) and imaged using a fluorescent microscope (A). Insets contain merged images showing TUNEL-positive cells. Upper corner inserts contain merged images from boxed areas. The data represent means expressed as the numbers of TUNEL-positive cells (B). $^{*}P<0.05$ versus WT mice; $^{\#}P<0.05$ versus CaBP-9k KO mice. Scale bars indicate 100 μm.

DISCUSSION

This is the first study to investigate the consequences of a total loss of function of CaBP-9k, CaBP-28k, and CaBP-9k/28k KO on apoptosis-related gene expression in uterine tissue. In this study, Bax protein expression was enhanced in the uteri of CaBP-28k and CaBP-9k/28k KO mice compared to WT and CaBP-9k mice. In addition, the expressions of caspase 3, 6, and 7 proteins were up-regulated in both CaBP-28k and CaBP-9k/28k KO mice, implying that the absence of calbindins may increase apoptosis-induced signaling and ER stress.

During the pre-implantation period, apoptotic uterine epithelial cell death occurs as part of the estrous cycle, and the sex steroid hormones E2 and P4 are key regulators of the estrous cycle [Dharma et al., 2001]. Apoptotic processes are directly responsible for the morphological changes that occur during the estrous cycle; uterine luminal epithelial cell weight increases from diestrus to estrus, and apoptosis peaks during metestrus and estrus. Uterine epithelial apoptosis appears to be regulated by steroid hormones [Sato et al., 1997]. The expression of apoptosis-related genes has been shown to be controlled by Bcl-2 family members, which include anti-apoptotic proteins such as Bcl-2 and Bcl-X, and pro-apoptotic proteins, such as Bax and Bak [Gross et al., 1999; Harris and Thompson, 2000; Antonsson, 2001]. Both Bcl-2 and Bax have been shown to be expressed in human endometrial tissue [McLaren et al., 1997], and expression of these proteins is higher in the secretory endometrium [Meresman et al., 2000]. In addition, caspases are well established as key mediators of apoptosis; caspases are divided into initiators (caspase 8 and 9) and effectors (caspases 3, 6, and 7) [Thornberry, 1998; Lakhani et al., 2006]. Initiator caspases exert regulatory roles by activating the downstream effectors of caspases 3, 6, and 7, which cleave various cellular substrates. Caspases are believed to target important proteins that regulate cell proliferation and survival [Cohen, 1997]. Caspase 3 is a key mediator of apoptosis and induces downstream caspase 6, which in turn induces the targeted cleavage of important structural proteins such as laminin and keratins [Caulin et al., 1997; Ruchaud et al., 2002]. In addition, poly(ADP-ribose) polymerase (PARP), a nuclear enzyme activated during DNA damage, is known to be cleaved by caspases 3 and 7 [Ohgushi et al., 1980]. Executioner caspases contribute to chromatin condensation and margination [Slee et al., 2001]. Therefore, caspases 3, 6, and 7 are key mediators of apoptosis and common downstream effectors of multiple apoptotic signaling pathways.

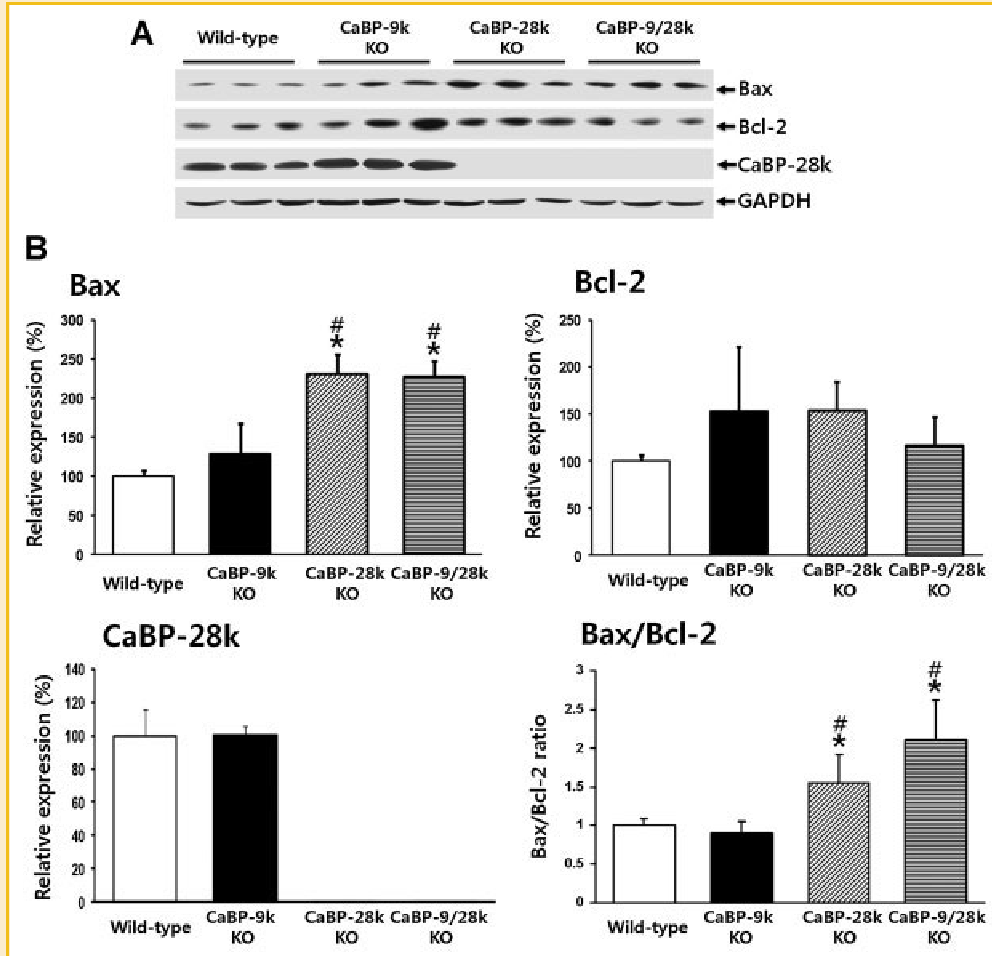

Fig. 2. Expression of apoptotic genes in the uteri of calbindin KO mice. Expression of Bax, Bcl-2, and CaBP-28k in calbindin KO mice was analyzed by Western blot (A) and results are represented as bar graphs. The Bax to Bcl-2 ratio was calculated from the expression of Bax and Bcl-2 (B). Protein expression was normalized to that of GAPDH and is shown as a percent of WT expression. Data represent the mean ± SEM of triplicate samples of three individual experiments. $^{*}P < 0.05$ versus WT mice; $^{\#}P < 0.05$ versus CaBP-9k KO mice.

Calcium is involved in the regulation of cell differentiation, proliferation, and apoptosis. In response to stimuli such as membrane depolarization, extracellular molecular signals, or intracellular messengers, the cytoplasmic concentration of calcium ions can increase from 10^{-7} M in the resting cell to 10^{-5} M in the activated cell, the result of either an influx of extracellular calcium or of intracellular calcium from the ER [Berridge et al., 2000, 2003; Gifford et al., 2007]. The ER is the site of the synthesis, folding, modification, and trafficking of secretory and cell-surface proteins. The ER lumen contains the largest store of intracellular calcium; thus, the ER also plays a critical role in maintaining calcium homeostasis. When the lumen of the ER is exposed to an oxidative environment, the disulfide bonds are formed, and the accepted folding of proteins for secretion displays on the cell surface. Because of its role in protein folding and transport, the ER is also rich in Ca^{2+}-dependent molecular chaperones, such as Grp78/Bip, Grp94, and calreticulin [Schroder and Kaufman, 2005; Xu et al., 2005]. Recent studies have suggested that the ER stress response is involved in the regulation of apoptosis. ER stress-induced apoptosis is mediated by the transcriptional activation of the transcription factor CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP). CHOP heterodimerizes with other C/EBP family members and thus induces apoptosis [Oliveira et al., 2009; Pino et al., 2009]. Thus, it is important to investigate the expression of the ER stress markers, BiP and CHOP. In this study, we observed significantly higher CHOP and BiP expression in CaBP-28k KO and CaBP-9k/28k double KO mice, suggesting that uterine CaBP-28k plays an important role as an inhibitor of ER stress. Thus, future work will attempt to correlate intracellular CaBP-28k activity with the expression of apoptosis-related genes and ER stress genes in CaBP-28k KO and CaBP-9k/28k double KO mice.

P4 induces CaBP-9k in the luminal epithelium before implantation but decreases levels of CaBP-9k at the site of embryo attachment during implantation in the murine uterus [Lee et al., 2003, 2005; An et al., 2003a,b, 2004a; Nie et al., 2005]. CaBP-9k and CaBP-28k are expressed in similar patterns during early pregnancy. Gene deletion with antisense oligonucleotides for CaBP-9k completely blocked the implantation process in CaBP-28k-null

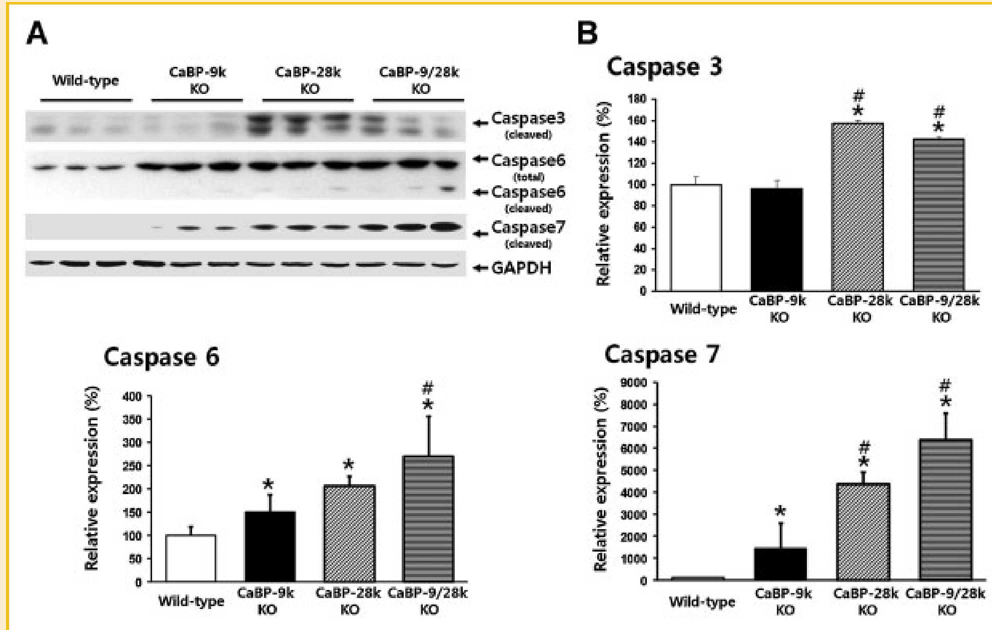

Fig. 3. Expression of activated caspases in the uteri of calbindin KO mice. Expression of caspase 3, 6, and 7 was measured by Western blot in calbindin KO mice (A) and results are represented as bar graphs (B). Protein expression was normalized to that of GAPDH and is shown as a percent of WT expression. Data represent the mean ± SEM of triplicate samples of three individual experiments. $^{*}P < 0.05$ versus WT mice; $^{\#}P < 0.05$ versus CaBP-9k KO mice.

mice Luu et al., 2004]. In our study, birth rates were significantly lower in CaBP-9k/28k KO mice than in CaBP-9k, CaBP-28k KO, or WT mice (data not shown). These observations indicate that CaBP-9k and CaBP-28k may not be essential for embryo implantation, and that the loss of CaBP-9k or CaBP-28k function can be compensated for by other implantation-related genes. A study using calbindin KO mice is further required to confirm role(s) of calbindins during implantation process. Recent studies have shown that apoptosis is regulated during implantation in the uterus [Spencer et al., 1996; Vinatier et al., 1996; Gosden and Spears, 1997; Jacobson et al., 1997; Milligan and Schwartz, 1997]. E2 has been shown to induce apoptotic signaling, while P4 inhibits it [Martin et al., 1970; Terada

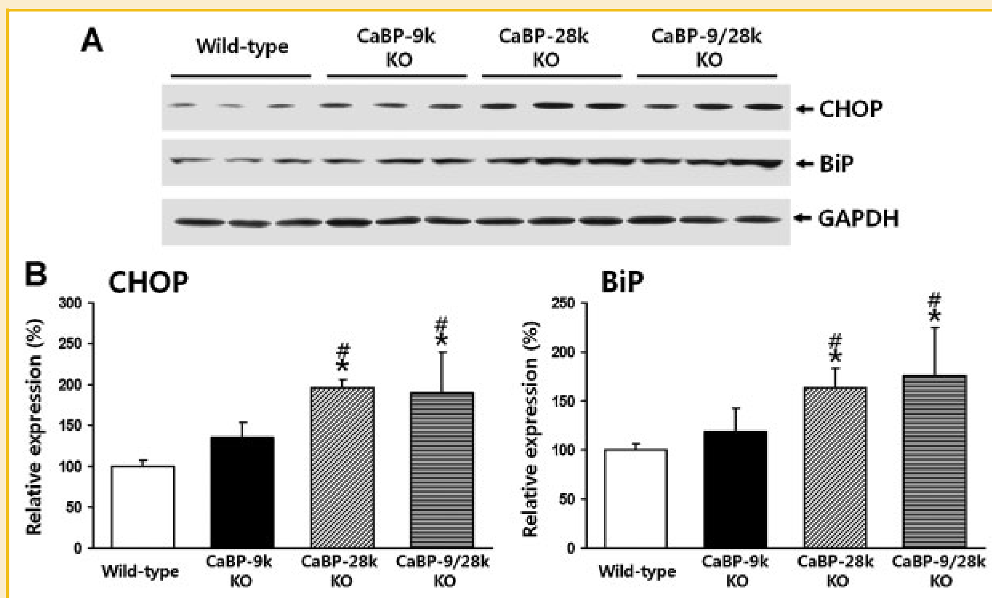

Fig. 4. Expression of ER stress genes in the uteri of calbindin KO mice. Expression of CHOP and BiP in calbindin KO mice was measured by Western blot analysis (A) and results are represented as bar graphs (B). Protein expression was normalized to that of GAPDH and is shown as a percent of WT expression. Data represent the mean ± SEM of triplicate samples of three individual experiments. $^{*}P < 0.05$ versus WT mice; $^{\#}P < 0.05$ versus CaBP-9k KO mice.

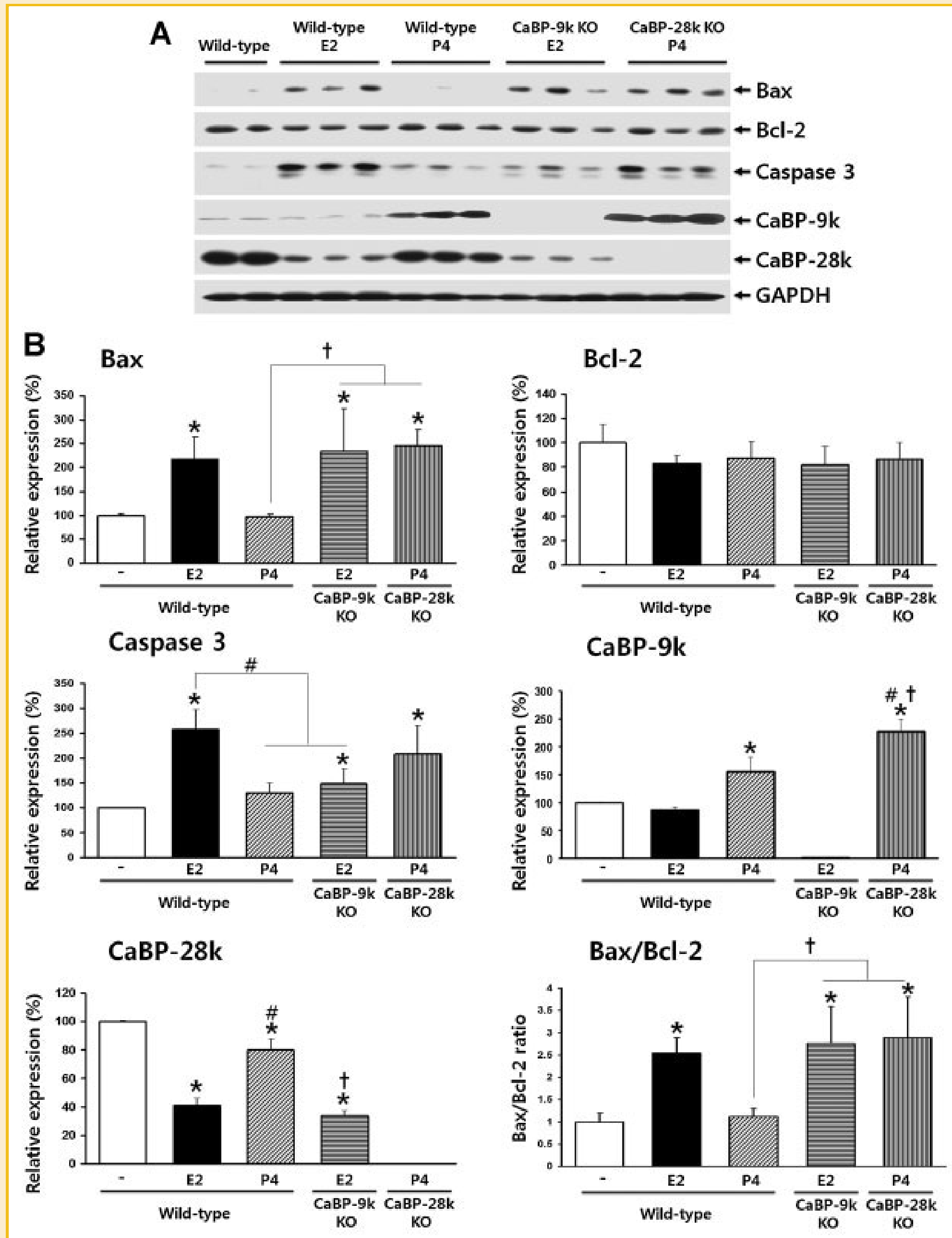

Fig. 5. Effects of steroid hormones on apoptosis-related gene expression in the uteri of calbindin KO mice. Expression of Bax, Bcl-2, caspase 3, CaBP-9k, and CaBP-28k was measured in calbindin KO mice by Western blot analysis (A) and results are represented as bar graphs. The ratio of Bax to Bcl-2 was calculated from the expression of Bax and Bcl-2 (B). Protein expression was normalized to that of GAPDH and is shown as percent expression of WT mice treated with 10% ethanol. Data represent the mean ± SEM of triplicate samples of three individual experiments. $^{*}P < 0.05$ versus WT mice; $^{\#}P < 0.05$ versus WT + E2; $^{\dagger}P < 0.05$ versus WT + P4.

et al., 1989], suggesting that apoptotic signaling is regulated by E2 and P4 in the murine uterus. In addition, uterine CaBP-9k expression is up-regulated by P4 [An et al., 2004b; Nguyen et al., 2005] and uterine CaBP-28k expression is down-regulated by E2 in immature mice [Opperman et al., 1992]. In this study, Bax and caspase 3 proteins were up-regulated by E2 in both WT and CaBP-9k KO mice, and by P4 in CaBP-28k KO mice, suggesting that CaBP-28k may block the induction of apoptotic signals in the murine uterus.

Recent studies have indicated that CaBP-28k blocks apoptotic processes that are induced by diverse signaling pathways. CaBP-28k has been demonstrated to inhibit apoptotic signaling in neural cells, renal cells, beta cells, osteoblasts, and endometrial cells, which suggests that CaBP-28k may inhibit apoptosis by buffering intracellular calcium and by interacting with the active form of caspase 3 [Guo et al., 1998; Bellido et al., 2000; Turner et al., 2000; Rabinovitch et al., 2001; Rintoul et al., 2001; Jung et al., 2011]. As further evidence of the ability of calcium-binding proteins to inhibit

apoptosis, melatonin-induced increased CaBP-9k expression in pituitary cells was shown to inhibit H_2O_2-mediated cell death [Yoo and Jeung, 2009, 2011].

This study is the first to demonstrate that CaBP-9k and CaBP-28k regulate apoptosis signaling pathways in the uterus using KO mice. Increased expression of apoptosis-related and ER stress-related genes was shown in the uteri of CaBP-28k KO and CaBP-9/28k KO mice. Our results suggest that CaBP-28k can protect against apoptosis and ER stress through gene regulation in uterine tissue. However, a further study warranties to elucidate the mechanism through which the expressions of ER stress-related genes may increase in CaBP-28k KO and CaBP-9/28k KO mice. In addition, this study demonstrated that the downstream effectors caspase 3, 6, and 7, and the ER stress-related genes CHOP and Bip, were up-regulated in the uterine tissue of CaBP-28k KO and CaBP-9k/28k double KO mice. A further study is necessary to clarify the upstream apoptotic signaling pathways and ER stress-related pathways in the uteri of immature and mature calbindin KO mice. The results of this study indicate that the expression of CaBP-28k down-regulates apoptotic signaling pathways and ER-stress-related genes in the uterine tissue of KO mice.

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