Expression of calbindin-D_{28k} is inversely correlated with proapoptotic gene expression in hydrogen peroxide-induced cell death in endometrial cancer cells

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Abstract. Calbindin-D_{28k} (CaBP-28k) is a calcium binding protein important for intracellular Ca^{2+} buffering and known to have anti-apoptotic properties in neurons, osteoblasts and male germ cells. Although endometrial cancer is a common invasive gynecologic malignancy, the involvement of uterine CaBP-28k in apoptotic signaling of endometrial cancer is poorly understood. The present study investigates the role of CaBP-28k in hydrogen peroxide (H,O_{2})-induced apoptotic signaling in human endometrial Ishikawa cells. The dose- and time-dependent effect of H,O_{2} on Bax, p53 and Bcl-2 expression was assessed by Western blot analysis. Treatment of cells with 1 mM H,O_{2} for 1 h induced an increase in Bax and p53 expression, but the expression of Bcl-2 was not affected by H,O_{2} treatment. Interestingly, overexpression of CaBP-28k inhibited cell death and caused a decrease in Bax, p53 and caspase 3 expression during H,O_{2}-induced apoptosis, suggesting that CaBP-28k blocks the up-regulation of apoptosis-related gene expression. siRNA knockdown of CaBP-28k resulted in an elevation of H,O_{2}-induced cell death and an increase in Bax, p53 and caspase 3, providing additional evidence that induction of the CaBP-28k gene might be associated with survival signaling during H,O_{2}-mediated cell death. Overall, these results suggest that CaBP-28k expression is inversely correlated with pro-apoptotic gene expression in human endometrial Ishikawa cells.

Introduction

Apoptosis (programmed cell death) is a genetically regulated cellular suicide mechanism essential for the removal of damaged or unwanted cells and the maintenance of tissue homeostasis in multicellular organisms. Apoptotic cells are characterized by their morphological alterations including condensation and fragmentation of nuclear chromatin, cytoplasmic contraction, and plasma membrane blebs (1,2). The role of apoptosis in the endometrium is not known. The process of apoptosis is regulated by several proteins, among them the members of the Bax, p53, Bcl-2 and caspase 3 protein families. Bcl-2 is a member of a group of anti-apoptotic proteins that can prevent or reduce cell death induced by a variety of stimuli. The Bax gene is a member of the Bcl-2 gene family and its protein product functions as a pro-apoptotic protein in the cytosol. Bax is present in a dimer form and higher Bax signals promote cell death. In addition, Bax heterodimerizes with Bcl-2 and neutralizes the anti-apoptotic function of Bcl-2. Bax is essential for the release of cytochrome c and the activation of caspases (3-5). In addition, the Bax gene promoter contains p53 consensus sequences and has been shown to be transcriptionally regulated by p53 (6,7). Therefore, p53 regulates apoptosis through a linear pathway involving Bax transactivation, cytochrome c release from mitochondria, and caspase 9 activation, followed by the activation of caspases 3, 6, and 7 (8). Caspase 3 is a key mediator of apoptosis and is a common downstream effector of multiple apoptotic signaling pathways (9).

Oxidative stress is induced by the accumulation of reactive oxygen species (ROS) and it is associated with the development of a variety of diseases such as neurodegenerative disorders (Alzheimer's disease and Parkinson's disease), cancer, cardiovascular disease and atherosclerosis (10). In addition, oxidative stress causes the influx of Ca^{2+} into the cytoplasm from the extracellular environment, and an elevated Ca^{2+} concentration in the cytoplasm results in Cr^{2+} influx into the mitochondria and nuclei. In the mitochondria, Ca^{2+} accelerates and disrupts normal metabolism leading to cell death. In nuclei, Ca^{2+} modulates gene transcription and regulates the activity of nucleases that control cell apoptosis (11-13). Therefore, calcium ions play an important role in apoptosis signaling pathways. Hydrogen peroxide (H,O_{2}) is produced during many intracellular reactions and can lead to apoptosis and necrosis in cells (14,15).

Calcium-binding proteins (calbindins) belong to a group of intracellular proteins with a high affinity for calcium and they include calmodulin, parvabumin, troponin C, calbindin-D_{9k}.

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CaBP-28k is expressed in a variety of organs and tissues, such as the intestine, kidney, pancreas, brain, bone and uterus (16-20). CaBP-28k is a member of the calmodulin superfamily of calcium binding proteins and contains six EF-hands that are critical for regulating the availability of calcium ions (Ca$^{2+}$) within cells (21). CaBP-28k has been shown to block apoptosis induced by different 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 release of cytochrome c (22). The Ca$^{2+}$-buffering effect of CaBP-28k in the kidney protects cells from apoptosis. Expression of CaBP-28k in HEK renal cells inhibited parathyroid hormone-induced apoptosis by buffering intracellular calcium (23,24). In addition, cytokine-induced apoptosis and necrosis of pancreatic β cells can be prevented by CaBP-28k (19). The effect of CaBP-28k in protecting osteoblasts against TNF and glucocorticoid-induced apoptosis involved the inhibition of caspase 3 activity (17,25).

Studies of apoptosis in the human endometrium revealed that apoptosis is rare in the proliferative endometrium, whereas the number of apoptotic cells progressively increases during secretory endometrium phases and peaks during the menstrual phase (26,27). The cyclical changes of the human endometrium during the menstrual cycle in preparation for blastocyst implantation suggest that apoptosis plays an important role in the function of endometrial cells (28). Moreover, human CaBP-28k protein expression reaches a peak in the luminal and glandular epithelium during the mid-secretory phase, at the onset of uterine receptivity to embryo implantation (29).

Although the Ishikawa cells used in most studies of endometrial function show functional similarities to the normal physiological responses of the endometrium (30,31), the regulation of apoptosis in the uterus, which is essential for the understanding of the physiology of the endometrium, has not been well studied. In particular, the role(s) of CaBP-28k expression during apoptosis signaling have not been examined in the human endometrium. In the present study, the exact role(s) of CaBP-28k in hydrogen peroxide (H$_2$O$_2$)-induced cell death/apoptosis was investigated in Ishikawa cells to understand the normal physiology of the human endometrium.

Materials and methods

Cell culture. Human endometrial Ishikawa cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL) at 37°C in 5% CO$_2$ and 95% air in a humidified cell incubator. Where indicated, media were supplemented with doses of H$_2$O$_2$ (0.25-2 mM) for 0-120 min.

Cell viability assay. Cell viability was determined using the Cell Counting kit-8 (Dojindo Laboratories, Tokyo, Japan). Briefly, Ishikawa cells were cultured in a 96-well plate (Corning Inc., Corning, NY, USA) at a density of 5x10$^4$ cells per well, with or without H$_2$O$_2$. The cells were washed and treated with the Cell Counting kit-8 solution, and the plate was incubated in the dark for an additional 4 h. The absorbance at 450 nm was read using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). Percent viability was calculated as follows: (absorbance of survived cells in the presence of H$_2$O$_2$/absorbance of control cells) x100.

TUNEL staining. Ishikawa cells were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. The cells were then washed in 1X PBS and permeabilized with 0.1% Triton X-100 in PBS with 0.1% sodium citrate for 20 min on ice. The cells were then washed twice with 1X PBS. DNA fragmentation in Ishikawa cell nuclei was detected by the TUNEL reaction mixture using the in situ Cell Death Detection kit (Roche Applied Science, Mannheim, Germany) for 1 h at 37°C in the dark. Cells were then washed three times for 5 min with 1X rinse buffer and incubated with DAPI (100 ng/ml) for nucleic acid staining. Fluorescence was detected using a fluorescent microscope (IX71 Inverted Microscope, Olympus, Japan). Positive control sections were treated with the same reagents but were pretreated with 1,000 U/ml DNase I (Takara Bio Inc., Shiga, Japan) in 50 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 1 mg/ml BSA for 10 min at room temperature prior to TUNEL assay. For negative controls, the sections were incubated with TUNEL label only.

Western blot analysis. Cells were harvested, washed two times with ice-cold PBS, and then resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, and 1 µg/ml chymostatin) and phosphatase inhibitors (5 mM Na$_2$VO$_4$, 5 mM NaF). Whole cell lysate was prepared using 20 strokes of a Dounce homogenizer, followed by centrifugation at 13,000 x g for 20 min at 4°C. Protein concentration was determined using the BCA assay (Sigma, St. Louis, CA, USA). Proteins (50 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with antibodies directed against the following proteins, as indicated: Bcl-2-associated protein (Bax) (1:1000), p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000), caspase 3 (Cell Signaling Technology, Beverly, MA, USA) (1:500), Bcl-2 (Cell Signaling Technology) (1:1000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000) (Assay Designs, Ann Arbor, MI, USA). Immunoreactive proteins were visualized by exposure to X-ray film. The protein bands were quantified by image-scanning, and optical density was measured using the Gel Doc EQ system (Bio-Rad Laboratories Inc.), then the data were corrected by background subtraction and normalized using GAPDH as an internal control.

Reverse transcriptase-PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the methods outlined in the protocol, and the concentration of total RNA was determined by measuring the absorbance at 260 nm. First strand complementary DNA (cDNA) was prepared by subjecting total RNA (1 µg) to reverse transcription using mMLV reverse transcriptase (Invitrogen Co.) and random primers (9-mers; Takara Bio Inc.). To determine the conditions for logarithmic phase PCR amplification for target mRNA, aliquots (1 µg) were amplified using different numbers of cycles. The GAPDH gene was
PCR-amplified to rule out the possibility of RNA degradation and was used to control for variations in mRNA concentration in the RT reaction. A linear relationship between PCR product band visibility and number of amplification cycles was observed for target mRNAs. The GAPDH and target genes were quantified using 28 and 30 cycles, respectively. The cDNA was amplified in a 20-µl PCR reaction containing 1 U Taq polymerase (iNtRON Bio Inc.), 2 mM dNTP, and 10 pM specific primers. PCR reactions were denatured at 95˚C for 30 sec, annealed at 58˚C for 30 sec, and extended at 72˚C for 30 sec. The oligonucleotide sequences for CaBP-28k were 5'-AGT GGT TAC CTG GAA GGA AAG G-3' (forward) and 5'-AGC AGG AAA TTC TCT TCT GTG G-3' (reverse); and for GAPDH, they were 5'-GGT GTG AAC CAT GAG AAG TAT GAC-3' (forward) and 5'-AGT AGA GGC AGG GAT GTG CT-3' (reverse). PCR products (8 µl) were fractionated on a 2.3% agarose gel, stained with ethidium bromide, and photographed under UV illumination. The photograph was scanned and analyzed using a Gel Doc EQ system (Bio-Rad Laboratories Inc.).

Small inhibitory RNA treatment. The CaBP-28k small inhibitory RNA (siRNA), 5'-UUU CUA UGA AGC CAC UGU GGU CAG U-3' (Invitrogen Corporation, San Diego, CA, USA), was designed using BLOCK-iT™ RNAi Designer (Invitrogen Corporation) and the CaBP-28k sequence (NM 004929.2). SiRNA knockdown of CaBP-28k was achieved by transfection of Ishikawa cells using Lipofectamine™ 2000 (Invitrogen Corporation). Prior to transfection, the medium was replaced with Opti-MEM (Gibco-BRL, Grand Island, NY, USA). Lipofectamine 2000-siRNA complexes were incubated for 20 min at room temperature, and then added to the cells, followed by incubation at 37˚C in a CO₂ incubator for 24 h.

CaBP-28k overexpression. For the construction of the CaBP-28k expression vector, a 786-bp fragment of CaBP-28k was generated by PCR using human uterine tissue cDNA as a template (forward primer, 5'-GGA TCC ATG GCA GAA TCC CAC CTG CA-3'; reverse primer, 5'-CTC GAG CTA GTT ATC CCC AGC ACA GA-3'). Amplified fragments were digested by...
BamHI and XhoI, and ligated into the recombinant pcDNA3.1 (+) vector. PCR products were analyzed by nucleotide sequencing (Genotech Co. Ltd., Daejeon, Korea). Ishikawa cells were transfected with the CaBP-28k expression vector (2 µg) using Lipofectamine 2000 (Invitrogen Corporation), as described for siRNA treatment.

Statistical analysis. The Tukey test was performed to determine significant differences between experimental groups. Statistical analyses were performed with Prism Graph Pad (v4.0; GraphPad Software Inc., San Diego, CA, USA). Values are expressed as the means ± standard deviation (SD). A P<0.05 was considered statistically significant.

Results

H₂O₂-induced apoptosis. Although H₂O₂ is known to induce apoptosis in various cells, its effect on endometrial cells has not been reported. The present results showed that H₂O₂ had a dose- and time-dependent cytotoxic effect on Ishikawa cells. The survival of cells treated with 1 or 2 mM H₂O₂ was decreased by approximately 32-77% at 1 h and 66-81% at 2 h, respectively, compared to control cells (Fig. 1A). The function of CaBP-28k was investigated by overexpression and siRNA knockdown of this calcium binding protein in Ishikawa cells. Overexpression of CaBP-28k was transfected with the CaBP-28k expression vector (2 µg) using Lipofectamine 2000 (Invitrogen Corporation), as described for siRNA treatment.

Figure 2. Dose- and time-dependent effect of H₂O₂ on the induction of Bax, p53 and Bcl-2 proteins. The expression levels of Bax, p53 and Bcl-2 induced by various concentrations of H₂O₂ were measured by immunoblot assay. Ishikawa cells were treated with H₂O₂ for 0, 0.25, 0.5, 1.0, 1.5 and 2 mM, and harvested 1 h after treatment (A). The cells were exposed to a single concentration (1 mM) of H₂O₂ and harvested at after 0, 15, 30, 60, 90 and 120 min (B). *p<0.05 (0 mM H₂O₂ and 0 min).

In addition, positive TUNEL staining was detected in all the positive control cells treated with DNase I. As shown in Fig. 1C, TUNEL staining revealed that treatment with 1 mM H₂O₂ resulted in an increase in the rate of apoptosis at 1 and 2 h, while no evidence of apoptosis by TUNEL assay was detected in negative controls and 0 mM H₂O₂, indicating that 1 mM H₂O₂ strongly induced apoptosis in Ishikawa cells.

H₂O₂-induced apoptosis is associated with Bax and p53 expression. The dose- and time-dependent effect of H₂O₂ on the induction of the apoptotic proteins Bax, p53 and Bcl-2 was examined by Western blot analysis. Dose response was assessed by incubating Ishikawa cells with H₂O₂ at concentrations of 0, 0.25, 0.5, 1.0, 1.5 and 2 mM; time response was evaluated with treatment times of 0, 15, 30, 60, 90 and 120 min. In the dose-response experiments, a significant increase in Bax protein expression was observed with 0.25-2 mM for 1 h as seen in Fig. 2A, and a significant increase in p53 expression was detected in response to 0.5 and 1 mM H₂O₂ for 1 h (Fig. 2A). In the time-response experiments, a significant increase in Bax expression was observed after 30-120 min of exposure to H₂O₂, as shown in Fig. 2B, and a significant increase in p53 expression was also observed in response to H₂O₂ treatment after 30-90 min (Fig. 2B). However, the expression pattern of the Bcl-2 protein was not altered in either a dose- or time-dependent manner. These results suggest that H₂O₂-induced apoptosis is mediated by Bax and p53 protein expression in Ishikawa cells.

Overexpression of CaBP-28k inhibited H₂O₂-induced apoptosis. To examine the effect of CaBP-28k on the expression of the apoptotic proteins Bax, p53, Bcl-2 and caspase 3 during H₂O₂-
induced apoptosis, CaBP-28k was stably overexpressed by transfection of Ishikawa cells with a full-length sequence. In the presence of H$_2$O$_2$, a significant increase in Bax, p53 and caspase 3 expression was observed in pcDNA3.1 (+) vector-transfected cells compared to vehicle controls, as seen in Fig. 3A. However, the expression of these proteins was downregulated by overexpression of CaBP-28k in the presence of H$_2$O$_2$ (Fig. 3A). No differences in Bcl-2 expression were detected during H$_2$O$_2$-induced apoptosis. The quantification of Bax, p53 and caspase 3 levels by densitometry indicates that overexpression of CaBP-28k may block H$_2$O$_2$-induced apoptosis by inhibiting the expression of these proapoptotic proteins, as shown in Fig. 3B.

Reduced expression of CaBP-28k enhanced H$_2$O$_2$-induced apoptosis. The function of CaBP-28k in Ishikawa cells was investigated by knocking down the protein through siRNA transfection. Reduced expression of CaBP-28k in siRNA-transfected cells was confirmed by reverse-transcriptase PCR (Fig. 4). Expression of the pro-apoptotic proteins Bax, p53 and caspase 3 was significantly increased in response to H$_2$O$_2$ treatment in Ishikawa cells in which CaBP-28k expression was knocked down in comparison to vehicle-transfected cells (Fig. 5). These proteins appeared to be constitutively expressed in CaBP-28k siRNA-transfected cells. There was no change in the expression pattern of the Bcl-2 protein in response to CaBP-28k siRNA transfection. These results indicate that CaBP-28k expression may be associated with survival signaling in H$_2$O$_2$-mediated oxidative cell death.

**Discussion**

The results of the present study show that H$_2$O$_2$ treatment at a concentration of 1 mM for 1 h induces apoptosis in human endometrial Ishikawa cells. The cytotoxic effects of H$_2$O$_2$ were confirmed by the detection of the typical signs of apoptosis such as nuclear fragmentation by TUNEL staining, as well as the induction of the expression of the pro-apoptotic proteins Bax and p53, which suggested that apoptosis signaling pathways are induced in the presence of H$_2$O$_2$ in Ishikawa cells.

Reactive oxygen species (ROS) such as H$_2$O$_2$ play important roles in the regulation of endometrial function. ROS are increased in the late secretory phase of the endometrium,
indicating an involvement in endometrial shedding by causing tissue damage (32,33). In addition, ROS mediate apoptotic cell death, as an increase in intracellular ROS levels correlates with cell death (34). Among the regulators of apoptotic signaling pathways, Bax, p53 and caspase 3 play important roles, and the expression of the genes encoding p53 and Bax has been shown to play an important regulatory role in apoptosis (35). Bcl-2 family proteins, including the pro-apoptotic protein Bax, are essential for the release of cytochrome c and the activation of caspases. Bax also heterodimerizes with Bcl-2 and neutralizes the anti-apoptotic function of Bcl-2 (3-5). The presence of both Bcl-2 and Bax in human endometrial tissue (36) and their increase in the secretory endometrium (37) have been reported in endometrial cancer cell lines, such as Hec-1A, KLE and RL95-2 (38). In addition, the Bax gene was shown to be a direct transcriptional target of p53 through its
interaction with the Bax gene promoter and in p53 mutants, which lost the ability to activate Bax transcription (6,39,40). Apoptosis is known to be mediated by the family of caspases, which are divided into initiator and effector caspases. Initiator caspases, such as caspases 8 or 9, exert regulatory roles by activating the downstream effector caspases 3, 6, or 7, which cleave various cellular substrates (41,42). Caspase 3 has been identified as being a key mediator of apoptosis in mammalian cells (43). Caspase 3 is considered an executor enzyme because it can be activated by other caspases and because it has catalytic specificity for a significant number of critical cellular substrates (44).

Calcium ions regulate many cellular functions including cell proliferation, differentiation and death (45,46). Calcium ions play a critical role in apoptosis, and the increase in intracellular calcium concentration has been shown to activate apoptotic pathways (47,48). In addition, an elevation or reduction in intracellular calcium ion levels can promote cell death through necrosis or apoptosis (49,50). The increase of intracellular calcium levels triggers the release of cytochrome c and the activation of caspase 3, leading to cell death (51,52). To determine the effects of oxidative stress, it is important to understand the mechanisms of oxidant-induced calcium dysregulation. The effect of H$_2$O$_2$ treatment on intracellular calcium levels was used in the present study as a cellular model to study the mechanism of apoptosis in response to an increase in intracellular calcium in the presence of H$_2$O$_2$. In addition, ER plays critical role toward maintenance of cellular calcium homeostasis. H$_2$O$_2$ induces both mitochondria-mediated and endoplasmic reticulum (ER) stress-mediated apoptosis (53).

The intracellular calcium level was increased in the presence of H$_2$O$_2$. Therefore, future study will focus on the expression of CaBP-28k correlated with an increase in ER stress in the presence of H$_2$O$_2$.

Recent evidence indicates that overexpression of calbindins prevents apoptosis. An increase of CaBP-9k expression prevented H$_2$O$_2$-mediated apoptosis in rat pituitary GH3 cells through a mechanism involving p53 interaction (54,55). CaBP-28k is a high-affinity calcium binding protein that is widely expressed in kidney, pancreas, brain, bone, and male and female reproductive tissues as a major target of 1,25 dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) action, and its major role is to facilitate 1,25(OH)$_2$D$_3$-dependent transcellular calcium transport. The three steps in transcellular calcium transport are apical uptake, intracellular movement and extrusion from the cell through an apical calcium channel identified in 1,25(OH)$_2$D$_3$ responsive epithelia [Transient receptor potential (TRP) family, TRPV6 and TRPV5] (56-58). The expression of calbindins is synchronized with the expression of epithelial calcium channels (59). CaBP-28k has a protective effect against cell death in neuronal cells, HEK renal cells, pancreatic β cells, osteoblastic cells and male germ cells, and plays a role in the regulation of apoptosis in these cells. In addition, the expression of CaBP-28k and the regulation of its activity are potentially important for the establishment of uterine receptivity (29). Exogenous CaBP-28k reduces oxidative stress and preserves mitochondrial function. Overexpression of calbindin prevents apoptosis in cultured neural cells expressing mutant presenlin-1 (22) and was found to protect HEK renal cells from PTH-induced apoptosis and cytotoxicity (24). The rate of apoptosis was significantly decreased in CaBP-28k-overexpressing pancreatic β cells treated with cytokines (19), and CaBP-28k overexpression protected osteoblasts against TNF-induced apoptosis through a mechanism involving the inhibition of caspase 3 activity (17). CaBP-28k also reduced the rate of apoptosis in germ cells in the Robertsonian heterozygous adult male mouse (60). Therefore, increasing intracellular CaBP-28k expression blocks apoptosis induced by different pro-apoptosis pathways.

The present study demonstrates, for the first time, that CaBP-28k expression inhibits apoptosis induced by H$_2$O$_2$ in human endometrial Ishikawa cells. Overexpression of CaBP-28k was found to have a protective effect against H$_2$O$_2$-induced cell death and to significantly decrease the expression of Bax, p53 and caspase 3. In addition, siRNA knockdown of CaBP-28k in Ishikawa cells caused a significant decrease in cell survival in H$_2$O$_2$-treated cells, concomitant with a significant increase in Bax, p53 and caspase 3 protein expressions. Our results are in close agreement with those of other studies showing that CaBP-28k can protect against cell death. Future study is required to focus on the interaction of CaBP-28k with apoptosis-related genes.

In summary, the results of the present study provide evidence that CaBP-28k protects against apoptosis by regulating the pro-apoptotic genes, Bax, p53 and caspase-3, in the presence of H$_2$O$_2$ in human endometrial cells.

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References


