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Overexpression of Regucalcin Suppresses Apoptotic Cell Death in Cloned Normal Rat Kidney Proximal Tubular Epithelial NRK52E Cells: Change in Apoptosis-Related Gene Expression

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Abstract The effect of regucalcin, a regulatory protein in intracellular signaling pathway, on cell death and apoptosis was investigated using the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin. NRK52E cells (wild type) and stable regucalcin (RC)/pCXN2 transfectants were cultured for 72 h in a medium containing 5% bovine serum (BS) to obtain subconfluent monolayers. After culture for 72 h, cells were further cultured for 24–72 h in a medium without BS containing either vehicle, tumor necrosis factor-α (TNF-α; 0.1 or 1.0 ng/ml of medium), lipopolysaccharide (LPS; 0.1 or 1.0 μg/ml), Bay K 8644 (10^{-9}–10^{-7} M), or thapsigargin (10^{-9}–10^{-7} M). The number of wild-type cells was significantly decreased by culture for 42–72 h in the presence of TNF-α (0.1 or 1.0 ng/ml), LPS (0.1 or 1.0 μg/ml), Bay K 8644 (10^{-7}–10^{-5} M), or thapsigargin (10^{-8} or 10^{-7} M). The effect of TNF-α (0.1 or 1.0 ng/ml), LPS (0.1 or 1.0 μg/ml), Bay K 8644 (10^{-7}–10^{-6} M), or thapsigargin (10^{-7} M) in decreasing the number of wild-type cells cultured for 24–72 h was significantly prevented in transfectants overexpressing regucalcin. Agarose gel electrophoresis showed the presence of low-molecular-weight deoxyribonucleic acid (DNA) fragments of adherent wild-type cells cultured with LPS (1.0 μg/ml), Bay K 8644 (10^{-7} M), or thapsigargin (10^{-8} M) for 24 h, and this DNA fragmentation was significantly suppressed in transfectants. DNA fragmentation in adherent cells was not seen by culture with TNF-α (1.0 ng/ml). TNF-α-induced decrease in the number of wild-type cells was significantly prevented by culture with caspase-3 inhibitor (10^{-8} M), while LPS- or Bay K 8644-induced decrease in cell number was significantly prevented by caspase-3 inhibitor or N ω-nitro-L-arginine methylester (NAME) (10^{-5} M), an inhibitor of nitric oxide (NO) synthase. Thapsigargin-induced decrease in cell number was not prevented in the presence of two inhibitors. Bcl-2 and Akt-1 mRNA levels were significantly increased in transfectants cultured for 24 h as compared with those of wild-type cells, while Apaf-1, caspase-3, or glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) mRNA expressions were not significantly changed in transfectants. Culture with TNF-α (1.0 ng/ml), LPS (1.0 μg/ml), Bay K 8644 (l0^{-7} M), or thapsigargin (10^{-8} M) caused a significant increase in caspase-3 mRNA levels in wild-type cells. LPS (1.0 μg/ml) significantly decreased Bcl-2 mRNA expression in the cells. Their effects on the gene expression of apoptosis-related proteins were not significantly changed in transfectants. This study demonstrates that overexpression of regucalcin has a suppressive effect on cell death and apoptosis induced by various factors which their action are mediated through many intracellular signaling pathways, and that it modulates the gene expression of apoptosis-related proteins. J. Cell. Biochem. 96: 1274–1285, 2005.

Key words: regucalcin; cell death; apoptosis; Bcl-2; Akt-1; kidney NRK52E cells

Liver has been shown to participate in the regulation of calcium metabolism through the hepatic bile system in rats, and the bile calcium excretion is increased by hormonal stimulation [Yamaguchi, 1980; review]. On the basis of this finding, it was recognized that a novel Ca^{2+}-binding protein, which differs from calmodulin, is present in the hepatic cytosol of rats [Yamaguchi and Yamamoto, 1978]. The name regucalcin was proposed for this Ca^{2+}-binding

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protein, which may regulate the Ca^{2+} effect on liver cell function [Yamaguchi, 1992; review]. The molecular weight of rat regucalcin is estimated as 33,388 Da composing of 299 amino acid residues [Shimokawa and Yamaguchi, 1993]. We have cloned cDNA for regucalcin from human, rat, mouse, rabbit, bovine, chicken, and toad livers [Misawa and Yamaguchi, 2000]. Comparison analysis reveals that the nucleotide sequences of regucalcin from seven vertebrate species were highly conserved in their coding region, and they had 69.9–91.3 identity [Misawa and Yamaguchi, 2000].

Regucalcin plays a role in maintaining cell homeostasis and function as a regulatory protein on intracellular signaling process in many cell types [Yamaguchi, 2000a,b, 2005; reviews]. The gene of regucalcin is highly conserved in vertebrate species [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The rat and human regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. Regucalcin are greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin messenger ribonucleic acid (mRNA) is mediated through Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999]. AP-1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1998, 1999; Misawa and Yamaguchi, 2002].

Regucalcin plays a role in the maintenance of intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinases and thyrosine kinases, protein phosphatases, NO synthase, protein synthesis, and the control of the enhancement of DNA and RNA syntheses in proliferative cells [Yamaguchi, 2000a,b, 2005; Tsurusaki and Yamaguchi, 2002a,b; Izumi et al., 2003]. Regucalcin may play a pivotal role in the regulation of cell function. Regucalcin has been shown to translocate to the nucleus of rat liver, and it has been demonstrated to regulate nuclear function in regenerating rat liver with proliferative cells [Tsurusaki et al., 2000]. Overexpression of regucalcin has a suppressive effect on cell proliferation and DNA synthesis in the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002]. Regucalcin may have a role as suppressor in the regulation of proliferation of liver cells [Yamaguchi and Daimon, 2005]. Moreover, overexpression of regucalcin has been shown to have a suppressive effect on cell death and apoptosis in the cloned rat hepatoma H4-II-E cells [Izumi and Yamaguchi, 2004a,b]. Regucalcin has been proposed to play a physiologic role in maintaining homeostasis of cellular response for cell stimulation [Yamaguchi, 2005; review]. Interestingly, regucalcin transgenic rats have been shown to induce bone loss and hyperglycemia [Yamaguchi et al., 2002, 2004], suggesting its pathophysiologic role.

Regucalcin is greatly expressed in liver, and its relatively higher levels are also found in kidney cortex [Yamaguchi and Isogai, 1993; Yamaguchi and Kurota, 1995]. Regucalcin may play a physiologic role in the regulation of cell function in kidney cortex cells which participate in reabsorption of calcium and other ions in kidney proximal tubular epithelial cells [Ng et al., 1982; Van Os, 1987]. Regucalcin has been shown to play a role in the regulation of intracellular Ca^{2+} transport [Kurota and Yamaguchi, 1997a,b; Xue et al., 2000], and this protein has an inhibitory effect on Ca^{2+}-dependent protein kinases [Kurota and Yamaguchi, 1997c], protein phosphatases [Morooka and Yamaguchi, 2002], and NO synthase [Ma and Yamaguchi, 2003] in the cytosol and nucleus of rat kidney cortex. Thus, regucalcin may have a role as a regulatory protein in kidney cells. The role of regucalcin in kidney cells has not been fully clarified, however.

This study was undertaken to determine the role of regucalcin in the cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro. Regucalcin has been shown to express in the NRK52E cells, and its mRNA expression has been demonstrated to be stimulated by parathyroid hormone or aldosterone, which targets kidney proximal tubular epithelial cells [Nakagawa and Yamaguchi, 2005a]. Recent study has demonstrated that cell proliferation is suppressed in NRK52E cells overexpressing regucalcin in vitro [Nakagawa et al., 2005]. Moreover, we examined the role of regucalcin in the regulation of cell death and apoptosis in NRK52E cells. We found that overexpression of regucalcin has a suppressive effect on cell death and apoptosis induced by various apoptotic factors in NRK52E cells, and that it enhances the expression of *Bcl-2* gene which suppresses apoptosis.

MATERIALS AND METHODS

Chemicals

Non-essential amino acid solution, bovine serum (BS), and penicillin-streptomycin solution (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Dulbecco's-modified Eagle's medium (DMEM), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), Bay K 8644, thapsigargin, N ω-nitro-L-arginine methylester (NAME) were obtained from Sigma Chemical Co. (St. Louis, MO). Caspase 3/CPP 32 inhibitor W-1 (caspase inhibitor) and other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan). Reagents used were dissolved in distilled water or ethanol, and some reagents were passed through ion-exchange resin to remove metal ions.

Regucalcin Transfectants

The cDNA encoding rat regucalcin was isolated and cloned into the pBluescript vector [Shimokawa and Yamaguchi, 1993]. The regucalcin cDNA contains *P*st I site downstream of the translational stop codon, and *P*st I site and an *Eco* RI upstream of the regucalcin cDNA. The *Eco* RI fragment (containing the complete coding cDNA) was cloned into the *Eco* RI site of the pCXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as regucalcin (RC)/pCXN2 [Misawa et al., 2002].

For transient transfection assay, the NRK52E cells were grown on 35-mm dishes to approximately 70% confluence. Each of RC/pCXN2 and pCXN2 vector alone was transfected into NRK52E cells using the synthetic cationic lipid components, a Tfx-20 reagent, according to the manufacturer's instructions (Promega) [Misawa et al., 2002]. At 48 h after transfection, cells were harvested and used for subsequent experiments. NRK52E cells were transfected with RC/pCXN2 vector alone using a Tfx-20 reagent. After 24 h, neomycin (1.0 mg/ml Geneticin G418, Sigma) was added to cultures for selection and cells were plated at limiting dilution. Multiple surviving clones were isolated, transferred to 35-mm dishes and grown in the medium without neomycin. Regucalcin was stably expressed in the transfectants. In experiments, transfectants were cultured for 24–72 h in DMEM containing 5% BS.

Cell Culture

The cloned normal rat kidney proximal tubular epithelial cells (NRK52E; 1×10^5 cells) [Rice et al., 2003] were maintained for 72 h in a DMEM supplemented with 5% BS, non-essential amino acid, and 50 U/ml, and 50 μg/ml streptomycin in humidified 5% CO_2/95% air 37°C to obtain subconfluent monolayers. After culture, cells were washed three times with phosphate-buffered saline (PBS), and the cells were incubated for 24, 48, or 72 h in DMEM without 5% BS in the absence or presence of various factors. Cells were washed three times with PBS after culture. Cells were collected to count cell numbers.

Cell Counting

After tripsinization of each of the culture dishes using 0.2% trpysin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min at 37°C, cells were collected and centrifuged in a PBS at 100*g* for 5 min. The cells were resuspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a Hemocytometer plate. For each dish, we took the average of two countings.

Analysis of DNA Fragmentation

The cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) and the transfectant of NRK52E cells with subconfluency were cultured for 24 h in a DMEM without 5% BS in the presence or absence of various factors. The culture supernatant was removed and adherent cells were then lysed in 10 mM Tris HCl, pH 7.4, 10 mM EDTA (neutralized), and 0.5% Triton X-100. DNA content was determined using the method of Ceriotti [1955]. Low-molecular-weight DNA fragments were separated by electrophoresis in 1.5% agarose gel [Preaux et al., 2002]. Gels were visualized by ethidium bromide staining with a UV transillimunator (Funakoshi Co. Ltd., Tokyo, Japan).

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. After culture, cells were washed three times with ice-cold PBS, and then cells were homogenized in buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl

alcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isoprepanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

RT-PCR was performed with a Titam™ One Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. The primers generated based on the published rat sequences. Primers for amplification Bcl-2 cDNA were: 5′-GCTACCGTCGCGACTTTGC-3′ (sense strand, positions 545–563 of cDNA sequence) and 5′-GCAGCGTCTTCAGAGACAGC-3′ (antisense strand, positions 867–886) [Sato et al., 1994]. The pair of oligonucleotide primers was designed to amplify a 342 bp sequence from the mRNA of rat Bcl-2. Primers for Akt-1 cDNA were: 5′-GGAGGGCTGGCTGCACAAACG-3′ (sense strand, positions 66–88 of cDNA sequence) and 5′-TCGTTCATGGTCACACGGTGCTTG-3′ (antisense strand, positions 465–488) [Chong et al., 2004]. The pair of oligonucleotide primers was designed to amplify a 424 bp sequence from the mRNA of rat Akt-1. Primers for Apaf-1 cDNA were: 5′-TCCAGCGGCAAGGACACAGACG-3′ (sense strand, positions 906–927 of cDNA sequence) and 5′-CAACCGCGTGCAAAGATTCTGCA-3′ (antisense strand, positions 1213–1235 of cDNA sequence). The pair of oligonucleotide primers was designed to amplify a 330 bp sequence from the mRNA of rat Apaf-1 [Chong et al., 2003]. Primers for caspase-3 cDNA were: 5′-CGCAATGGTACCGATGTCGATGC-3′ (sense strand, positions 246–268 of cDNA sequence) and 5′-GCAGTCCAGCTCTGTACCTCGGCA-3′ (antisense strand, positions 543–566) [Juan et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 321 bp sequence from the mRNA of rat caspase-3. Glyceroaldehyde-3-phosphase dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input. G3PDH cDNA were 5′-GATTGGCCGTATCGGACGC-3′ (sense strand) and 5′-CTCCTTGGAGGCCATGTAGG-3′ (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977 bp sequence from the mRNA of rat G3PDH. RT-PCR was performed using reaction mixture (20 μl) containing 2 or 4 μg of total RNAs, supplied RT-TCR buffer, Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50°C for 30 min, and then amplified for 30 cycles under the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 56°C, and extension for 60 s at 62.0–63.4°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Image density was quantified with a FluoroImager SI (Amersham Phamacia Biotech).

Statistical Analysis

Data were expressed as the mean ± SEM. The significance of differences between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. A *P*-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Effect of TNF-α, LPS, Bay K 8644, or Thapsigargin in Kidney NRK52E Cells Overexpressing Regucalcin

The cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type), pCXN2 transfected cells (mock type), or stable regucalcin (RC)/pCXN2 transfectants were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers. The proliferation of the cells was significantly suppressed in transfectants cultured for 72 h, as shown previously [Nakagawa et al., 2005]. After culture for 72 h, cells were changed to medium not containing BS in the presence or absence of various factors that can induce cell death and apoptosis, and further cultured for 24–72 h. The expression of regucalcin in the cells was remarkable in transfectants (data not shown), as shown previously [Nakagawa et al., 2005].

NRK52E cells with subconfluent monolayers were cultured for 24, 48, or 72 h in a medium without BS containing either vehicle, TNF-α (0.1 or 1.0 ng/ml) (Fig. 1), LPS (0.1 or 1.0 μg/ml) (Fig. 2), Bay K8644 (10^{-7}–10^{-5} M), or thanpsigargin (10^{-9}–10^{-7} M). The number of wild-type cells was significantly decreased by culture of 24–72 h in the presence of TNF-α(0.1 or 1.0 ng/ml), LPS (0.1 or 1.0 μg/ml), Bay K 8644 (10^{-7}–10^{-5} M), or thapsigargin (10^{-9}–10^{-7} M). These

decreases were also observed in mock-type cells (data not shown). The effect of TNF-α (0.1 or 1.0 ng/ml) in decreasing the number of NRK52E cells (wild type) was not observed in stable RC/pCXN2-transfected cells cultured for 24, 48, or 72 h (Fig. 1). The effect of LPS (0.1 or 1.0 μg/ml) inducing cell death was not seen in transfectants cultured for 24 or 48 h (Fig. 2). With culture for 72 h, cell number of transfectants was significantly decreased in the presence of LPS (0.1 or 1.0 μg/ml). The effect of Bay K 8644 (10^{-7} or 10^{-6} M) in decreasing the number of wild-type cells was significantly prevented in transfectants cultured for 24 or 48 h (Fig. 3). Culture with Bay K8644 (10^{-6} or 10^{-5} M) for 72 h caused a significant decrease in cell number of transfectants. The effect of thapsigargin (10^{-9} M) in inducing cell death was not seen in transfectants cultured for 24–72 h (Fig. 4).

Effect of TNF-α, LPS, Bay K 8644, or Thapsigargin on DNA Fragmentation in Kidney NRK52E Cells Overexpressing Regucalcin

NRK52E wild-type cells and transfectants with subconfluent monolayers were cultured for 24 h in a medium without BS containing

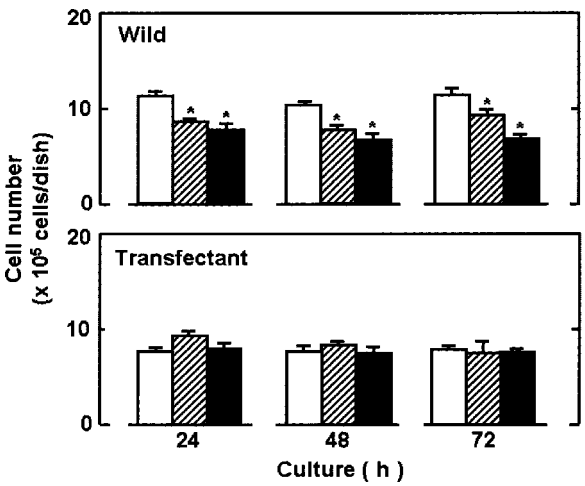

Fig. 1. Effect of tumor necrosis factor-α (TNF-α) on the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle or TNF-α (0.1 or 1.0 ng/ml of medium). After medium change, cells were incubated for 24, 48, or 72 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the control value. White bars, control (none); hatched bars, TNF-α (0.1 ng/ml); black bars, TNF-α (1.0 ng/ml).

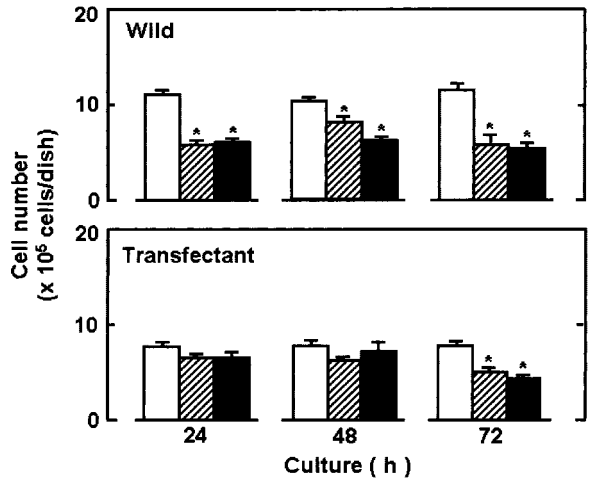

Fig. 2. Effect of lipoplysaccharide (LPS) on the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle or LPS (0.1 or 1.0 μg/ml of medium). After medium change, cells were incubated for 24, 48, or 72 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the control value. White bars, control (none); hatched bars, LPS (0.1 μg/ml); black bars, LPS (1.0 μg/ml).

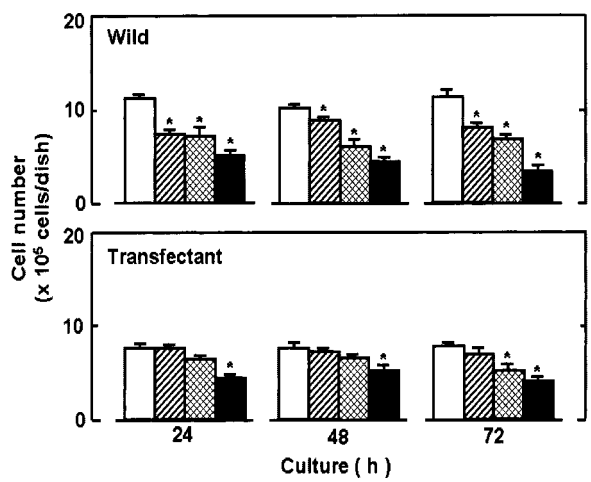

Fig. 3. Effect of Bay K 8644 on the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle or Bay K8644 (10^{-7}–10^{-5} M). After medium change, cells were incubated for 24, 48, or 72 h and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the control value. White bars, control (none); hatched bars, Bay K8644 (10^{-7} M); double hatched bars, Bay K8644 (10^{-6} M), black bars, Bay K8644 (10^{-5} M).

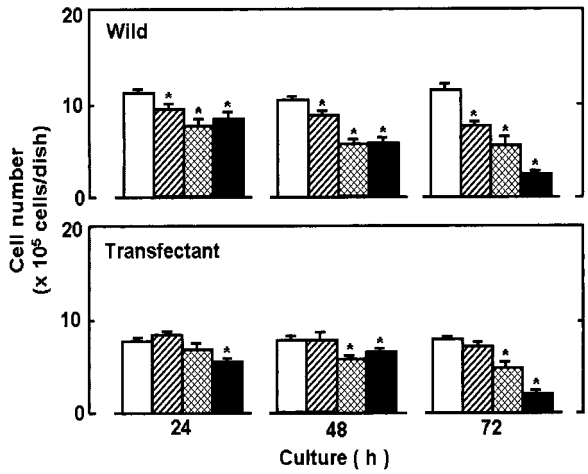

Fig. 4. Effect of thapsigargin on the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle or thapsigargin (10^{-9}–10^{-7} M). After medium change, cells were incubated for 24, 48, or 72 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the control value. White bars, control (none); hatched bars, thapsigargin (10^{-9} M); double hatched bars, thapsigargin (10^{-8} M); black bars, thapsigargin (10^{-7} M).

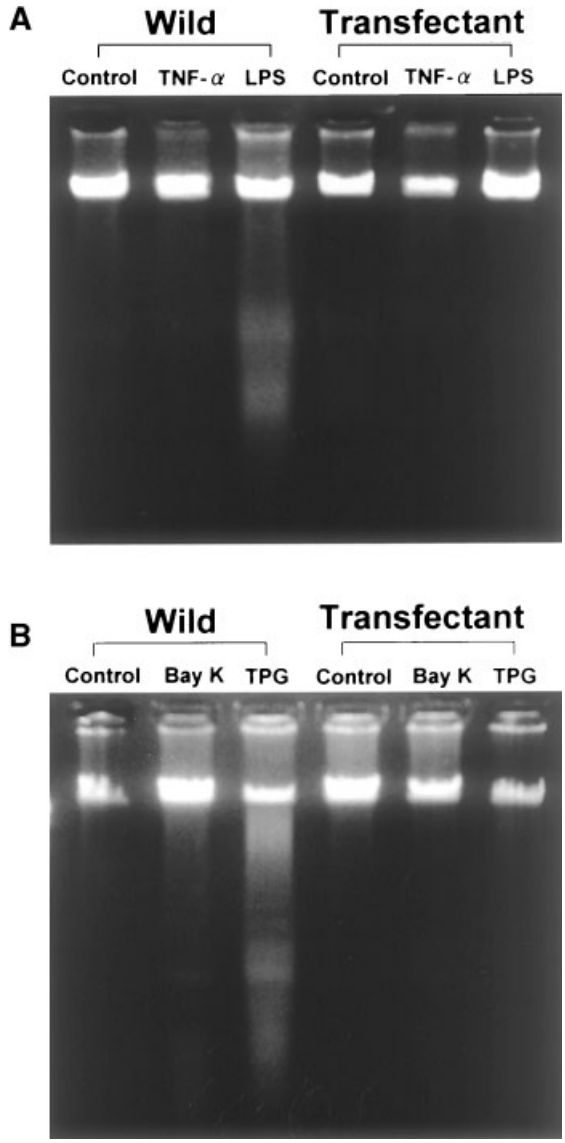

Fig. 5. Effect of TNF-α, LPS, Bay K8644, or thapsigargin on DNA fragmentation in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type; W) or regucalcin (RC)/pCXN2-transfected cells (T). Cells were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle, TNF-α (1.0 ng/ml), LPS (1.0 μg/ml), Bay K8644 (10^{-7} M), or thapsigargin (10^{-8} M). After medium change, cells were incubated for 24 h and the lysate (containing 1.0 μg of DNA) of adherent cells was applied to agarose gel. The figure shows one of four experiments with separate samples. **A**: Represents the result of TNF-α or LPS. **B**: Represents the result of Bay K8644 or thapsigargin.

either vehicle, TNF-α (1.0 ng/ml), LPS (1.0 μg/ml), Bay K 8644 (10^{-7} M), or thapsigargin (10^{-8} M) (Fig. 5). Adherent cells were lysed and then the lysate was separated by electrophoresis in agarose gel. Culture with LPS, Bay K8644, or thapsigargin caused DNA fragmentation in wild-type cells. The effect of those factors on DNA fragmentation was significantly prevented in transfectants. DNA fragmentation in adherent cells was not observed by culture with TNF-α (1.0 ng/ml). Likewise, the effect of LPS, Bay K8644, or thapsigargin in stimulating DNA fragmentation in adherent cells cultured for 48 h was significantly abolished in transfectants (data not shown).

Effect of Caspase Inhibitor or NAME in Kidney NRK52E Cells Overexpressing Regucalcin

NRK52E cells (wild type) or transfectants with subconfluent monolayers were cultured for 24 h in a medium without BS containing either vehicle, TNF-α(1.0 ng/ml), LPS (1.0 μg/ml), Bay K 8644 (10^{-7} M), or thapsigargin (10^{-8} M) in the presence or absence of caspase inhibitor (10^{-8} M) or NAME (10^{-5} M), an inhibitor of nitric oxide (NO) synthase, with an effective concentration on enzyme activity (Fig. 6). The effect of TNF-α(1.0 ng/ml) in decreasing cell number of NRK52E wild-type

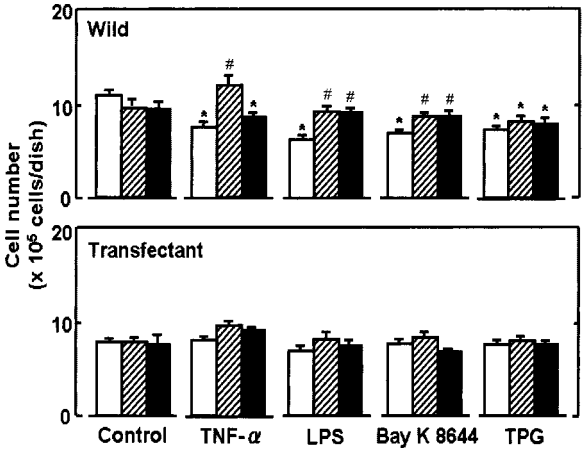

Fig. 6. Effect of caspase inhibitor or NAME on various factors-induced decrease in the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle, TNF-α (1.0 ng/ml of medium), LPS (1.0 μg/ml), Bay K8644 (10^{-7} M), or thapsigargin (10^{-8} M) in the presence or absence of caspase inhibitor (10^{-8} M) or NAME (10^{-5} M). After medium change, cells were incubated for 24 h and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$ compared with the control value. White bars, control (none); hatched bars, caspase inhibitor; black bars, NAME.

cells was not seen in the presence of caspase inhibitor (10^{-8} M), while it was observed in the presence of NAME (10^{-5} M). The effect of LPS (1.0 μg/ml) in decreasing wild-type cells was significantly changed by caspase inhibitor (10^{-8} M) or NAME (10^{-5} M). Cell number of transfectants was not significantly changed by caspase inhibitor (10^{-8} M) or NAME (10^{-5} M) in the presence or absence of TNF-α(1.0 ng/ml) or LPS (1.0 μg/ml). Bay K 8644 (10^{-7} M)-induced decrease in the number of wild-type cells was significantly prevented in the presence of caspase inhibitor or NAME which did not have an effect on transfectants. The effect of thapsigargin (10^{-8} M) in decreasing the number of wild-type cells was not significantly changed in the presence of caspase inhibitor or NAME.

Change in Gene Expression of Apoptosis-Related Protein in Kidney NRK52E Cells Overexpressing Regucalcin

The change in mRNA expression of apoptosis-related proteins in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or stable RC/pCXN2-transfected cells with subconfluent monolayers were cultured for 24 h in a medium without BS containing either vehicle, TNF-α(1.0 ng/ml), LPS (1.0 μg/ml), Bay K8644 (10^{-7} M), or thapsigargin (10^{-8} M). Total RNAs were extracted from adherent cells. The result of RT-PCT analysis using specific primers showed that Bcl-2 and Akt-1 mRNA levels were significantly increased in transfectants as compared with those of wild-type cells (Fig. 7). The expression of Apaf-1, caspase-3, or G3PDH mRNAs was not significantly changed in transfectants (Fig. 7).

Culture with TNF-α (1.0 ng/ml) caused a significant increase in caspase-3 mRNA levels in wild-type cells (Fig. 8). LPS (1.0 μg/ml) significantly increased caspase-3 mRNA levels in wild-type cells, and it significantly decreased Bcl-2 mRNA expression in the cells. Meanwhile,

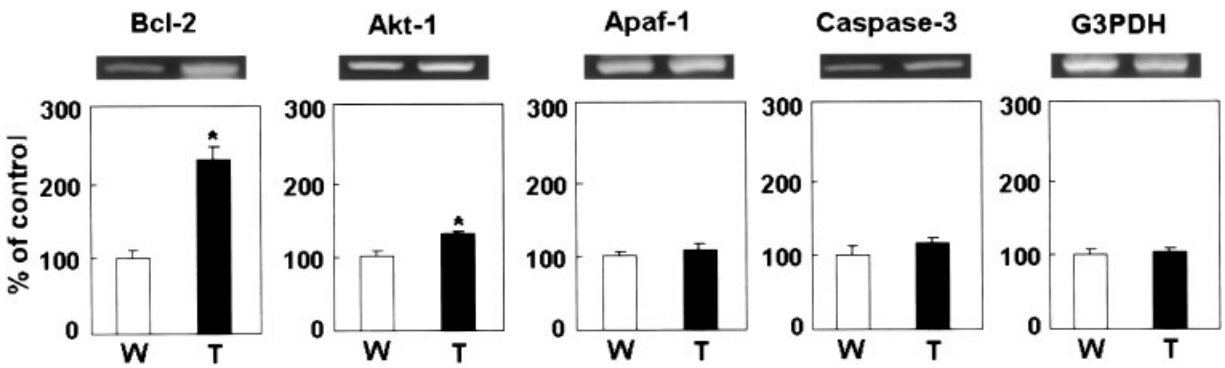

Fig. 7. Change in mRNA expression of various proteins which are related to cell death and apoptosis in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type; W) or regucalcin (RC)/pCXN2-transfected cells (T). Cell were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS. After medium change, cells were cultured for 24 h. Total RNAs (2 μg) extracted form the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA levels were indicated as % control (mean ± SEM of five experiments). $^{*}P < 0.01$ compared with the control value.

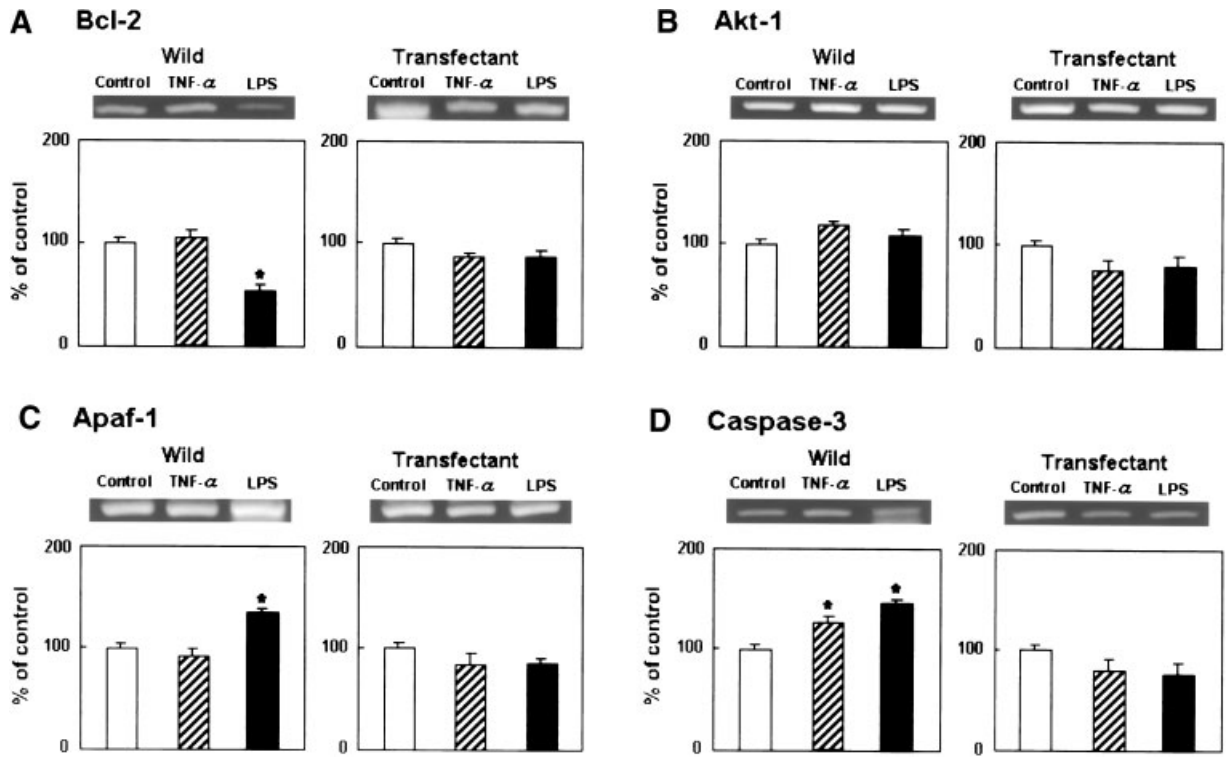

Fig. 8. Effect of TNF-α or LPS on mRNA expression of various proteins which are related to cell death and apoptosis in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cell were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS. After medium change, cells were cultured for 24 h. Total RNAs (2 μg) extracted form the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA levels were indicated as % control (mean ± SEM of five experiments). $^*P < 0.01$ compared with the control value.

the expression of Bcl-2, Akt-1, or caspase-3 mRNAs was not significantly changed in transfectants cultured with TNF-α (1.0 ng/ml) or LPS (1.0 μg/ml) (Fig. 8). Apaf-1 mRNA expression was significantly decreased in transfectants cultured with LPS (1.0 μg/ml), while it was not changed in transfectants cultured with TNF-α(1.0 ng/ml). Thus, the effect of TNF-α or LPS on the gene expression of apoptosis-related proteins was not significantly changed in transfectants.

The effect of Bay K 8644 or thapsigagin on gene expression of apoptosis-related proteins is shown in Figure 9. Culture with Bay K 8644 (10^{-7} M) or thapsigargin (10^{-8} M) caused a significant increase in caspase-3 mRNA levels in wild-type cells. This increase was completely prevented in transfectants. The expression of Bcl-2, Akt-1, or Apaf-1 mRNAs in wild-type cells or transfectants was not significantly changed in the presence of Bay K 8644 (10^{-7} M) or thapsigargin (10^{-8} M). G3PDH mRNA levels in wild-type cells or transfectants were not significantly changed by culture with Bay K 8644 (10^{-7} M) or thapsigargin (10^{-8} M) (data not shown).

DISCUSSION

Overexpression of regucalcin has been shown to have a suppressive effect on cell death and apoptosis induced by TNF-α, LPS, Bay K 8644, or thapsigargin, which induces apoptotic cell death mediated by various intracellular signaling mechanism in the cloned rat hepatoma H4-II-E cells [Izumi and Yamaguchi, 2004a,b]. We found, moreover, that their factors-induced apoptotic cell death is suppressed in the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin. Thus, regucalcin demonstrated to have a suppressive effect on various factors-induced apoptotic cell death in different cell types.

Culture with LPS, Bay K8644, or thapsigargin caused DNA fragmentation in wild-type cells. The effect of those factors on DNA fragmentation was significantly prevented in

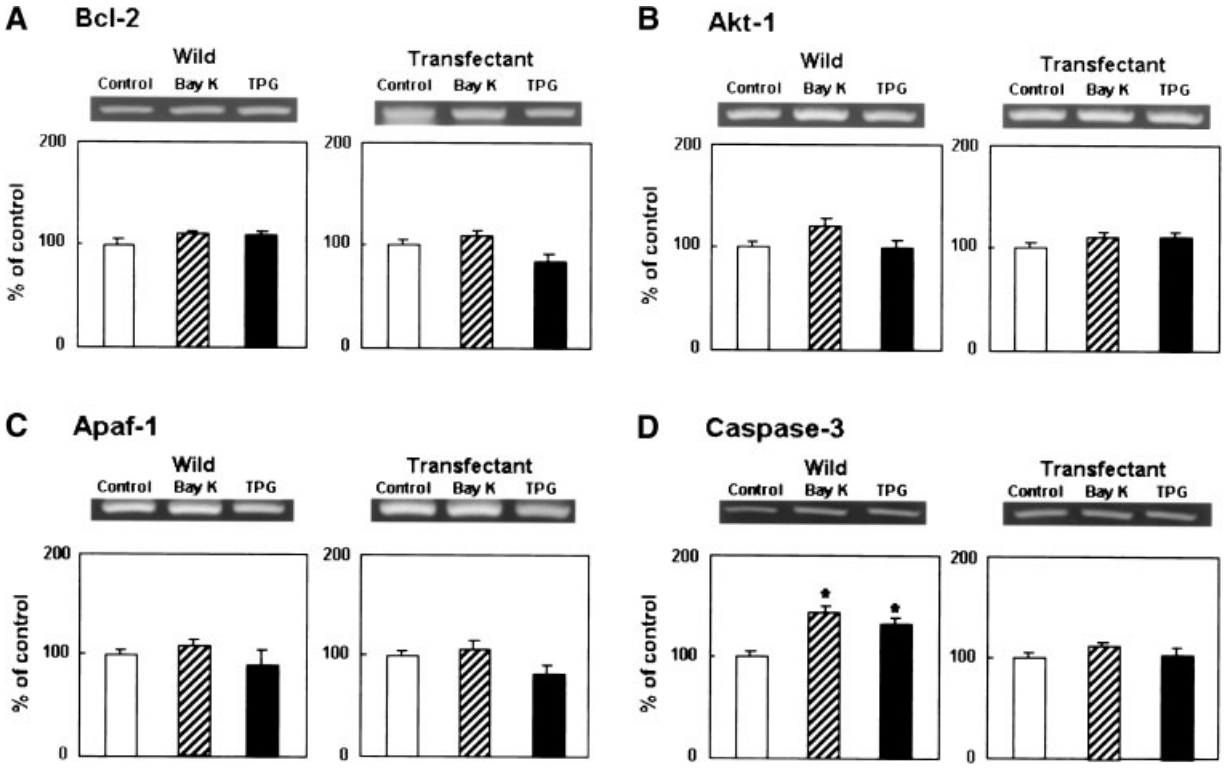

Fig. 9. Effect of Bay K 8644 or thapsigargin on mRNA expression of various proteins which are related to cell death and apoptosis in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cell were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle, Bay K 8644 (Bay K; 10^{-7} M), or thapsigargin (TPG; 10^{-8} M). After medium change, cells were cultured for 24 h. Total RNAs (2 μg) extracted form the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA levels were indicated as % control (mean ± SEM of five experiments). $^{*}P < 0.01$ compared with the control value.

transfectants. However, DNA fragmentation in adherent cells was not observed by culture with TNF-α (1.0 ng/ml). TNF-α may have a potent effect on apoptotic cell death in adherent cells of wild type. If TNF-α stimulates DNA fragmentation in adherent cells and the cell death is induced during short time, we cannot observe DNA fragmentation in the cells.

The mechanism by which TNF-α, LPS, Bay K8644, or thapsigargin induces apoptotic cell death in the cloned rat hepatoma H4-II-E cells has not been fully determined. TNF-α, LPS-, or Bay K8644-induced cell death in NRK52E cells was significantly prevented in the presence of caspase-3 inhibitor in NRK52E cells. LPS- or Bay K-8644-induced cell death was significantly blocked by NAME, an inhibitor of NO synthase, in the cells. Thapsigargin-induced cell death was not significantly suppressed in the presence of caspase-3 inhibitor or NAME. NO may be important as a signaling factor in many cells [Lowenstein et al., 1994], and it plays a role in apoptosis of hepatoma cells [Liu et al., 2000]. NO mediates apoptosis by D-galactosamine in a primary culture of rat hepatocytes [Abou-Elella et al., 2002]. NO is produced by NO synthase which is activated by Ca^{2+}/calmodulin. Bay K8644 is an agonist of Ca^{2+} entry in cells. Calcium entry into cells induces cell death [Cano-Abad et al., 2001; Pereira et al., 2002]. Overexpression of regucalcin had a suppressive effect on apoptotic cell death induced by TNF-α, LPS, Bay K 8644, or thapsigargin in kidney NRK52E cells. The effect of regucalcin in suppressing apoptotic cell death may be mediated through its action on many intracellular signaling pathways in kidney NRK52E cells.

Regucalcin has been demonstrated to have an inhibitory effect on Ca^{2+}-dependent protein kinases [Kurota and Yamaguchi, 1997c], protein phosphatases [Morooka and Yamaguchi, 2002], and NO synthase [Ma and Yamaguchi, 2003] in the cytoplasm and nucleus of rat kidney

cortex. These signal pathways may be inhibited in kidney NRK52E cells overexpressing regucalcin. Moreover, regucalcin has been shown to maintain intracellular Ca^{2+} homeostasis in rat kidney cortex cells [Kurota and Yamaguchi, 1997a,b; Xue et al., 2000]. This may also contribute to the suppressive effect of regucalcin on apoptotic cell death, since DNA fragmentation is activated by accumulation of nuclear Ca^{2+} [Yamaguchi and Sakurai, 1991].

Bcl-2 is a suppressor in apoptotic cell death [Vogelstein et al., 2000]. Apaf-1 participates in activation of caspase-3 [Zou et al., 1997]. Akt-1 involves in survival signaling pathway for cell death [Widmann et al., 1988]. Overexpression of regucalcin caused a remarkable elevation of Bcl-2 mRNA expression in NRK52E cells, and it slightly stimulated Akt-1 mRNA expression in the cells. Apaf-1, caspase-3, or G3PDH mRNA expressions were not significantly altered in transfectants. Presumably, the enhancement of Bcl-2 mRNA expression contributes to the suppression of apoptotic cell death in NRK52E cells overexpressing regucalcin. It is speculated that regucalcin plays a role in the regulation of Bcl-2 gene expression in NRK52E cells.

TNF-α significantly enhanced the expression of caspase-3 mRNA in NRK52E cells. This enhancement was found to suppress in transfectants. The result suggests that the mechanism by which regucalcin suppresses TNF-α-induced cell death is partly related to the decrease in caspase-3 mRNA expression in transfectants.

The presence of LPS caused a significant decrease in Bcl-2 mRNA levels in NRK52E cells, suggesting that this decrease is partly related to LPS-induced cell death. The enhancement of Bcl-2 mRNA expression induced by overexpression of regucalcin was also seen in the presence of LPS. Moreover, LPS-stimulated expression of Apaf-1 mRNA was significantly suppressed by overexpression of regucalcin. This may partly involve in the suppression of LPS-induced cell death in NRK52E cells overexpressing regucalcin.

Culture with Bay K 8644 (10^{-7} M) or thapsigargin (10^{-8} M) was found to cause a significant increase in caspase-3 mRNA levels in wild-type cells, indicating that the increased gene expression partly contributes to inducing apoptotic cell death. This increase was completely prevented in transfectants. Regucalcin may have a suppressive effect on caspase-3 mRNA expression enhanced by Bay K 8644 or thapsigargin in NRK52E cells.

Thus, regucalcin was found to regulate the expression of Bcl-2, caspase-3, and Akt-1 mRNAs in the cloned normal rat kidney NRK52E cells using RT-PCR with specific primers. The change in protein levels, however, remains to be elucidated.

Regucalcin has been shown to translocate into the nucleus of rat liver cells [Tsurusaki et al., 2000]. Regucalcin binds to nuclear protein and DNA, and it modulates tumor-related gene expression in the liver cells [Tsurusaki and Yamaguchi, 2004]. Whether regucalcin can directly modulate gene expression in the nucleus of kidney cells is unknown. However, it is possible that regucalcin translocates into the nucleus and it modulates gene expression in kidney NRK52E cells.

Whether regucalcin plays a physiologic role in kidney cells is unknown. However, overexpression of regucalcin was found to have a suppressive effect on apoptotic cell death induced by various factors (including TNF-α, LPS, Bay K 8644, or thapsigargin) in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. It has been reported that toxic factors induce renal failure due to stimulating apoptotic cell death [Dieguez-Acuna et al., 2004]. Regucalcin may have a role as a suppressor in the development of apoptotic cell death in kidney proximal tubular epithelial cells. Presumably, regucalcin plays a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation.

In conclusion, it has been demonstrated that overexpression of regucalcin has a suppressive effect on cell death and apoptosis induced by various factors which are involved in various intracellular signaling pathway in the cloned normal rat kidney proximal tubular epithelial NRK52E cells, and that it modulates the gene expression of apoptosis-related proteins.

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