Journal of Cellular Biochemistry 100:1178–1190 (2007)

Overexpression of Regucalcin Suppresses Cell Response for Tumor Necrosis Factor-α or Transforming Growth Factor-β1 in Cloned Normal Rat Kidney Proximal Tubular Epithelial NRK52E Cells

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Abstract The regulatory role of regucalcin on cell responses for tumor necrosis factor-α (TNF-α) or transforming growth factor-β1 (TGF-β1) was investigated using the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin. NRK52E cells (wild type) and stable regucalcin (RC)/pCXN2-transfected cells (transfectant) were cultured for 72 h in a medium containing 5% bovine serum (BS) to obtain subconfluent monolayers. After culture, cells were further cultured for 24–72 h in medium without BS containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-β1 (1.0 or 5.0 ng/ml). Culture with TNF-α or TGF-β1 caused a significant decrease in the number of wild-type cells. This decrease 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 TNF-α (1.0 ng/ml) or TGF-β1 (5.0 ng/ml). This DNA fragmentation was significantly suppressed in transfectants. TNF-α- or TGF-β1-induced cell death was significantly prevented in culture with caspase-3 inhibitor (10^{-8} M). Nitric oxide (NO) synthase activity in wild-type cells was significantly increased by addition of calcium chloride (10 μM) and calmodulin (5 μg/ml) into the enzyme reaction mixture. This increase was significantly suppressed in transfectants. Culture with TNF-α caused a significant increase in NO synthase activity in wild-type cells. The effect of TNF-α was not seen in transfectants. Culture with TGF-β1 did not cause a significant increase in NO synthase activity in wild-type cells and transfectants. Culture with TNF-α or TGF-β1 caused a remarkable increase in α-smooth muscle actin in wild-type cells. This increase was significantly prevented in transfectants. The expression of Smad 2 or NF-κB mRNAs was significantly increased in transfectants as compared with that of wild-type cells. Smad 3 or glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) mRNA expression was not significantly changed in transfectants. NF-κB mRNA expression in wild-type cells was significantly increased with culture of TNF-α. Smad 2 mRNA expression was significantly enhanced in wild-type cells cultured with TGF-β1. These effects of TNF-α or TGF-β1 were not significantly enhanced in transfectants. This study demonstrates that overexpression of regucalcin has suppressive effects on cell responses which are mediated through intracellular signaling pathways of TNF-α or TGF-β1 in kidney NRK52E cells. J. Cell. Biochem. 100: 1178–1190, 2007.

Key words: regucalcin; TNF-α; TGF-β1; actin; smad 2; NF-κB; kidney NRK52E cells

Regucalcin was discovered as a novel Ca^{2+}-binding protein not including the EF-hand motif in the liver cytosol of rats [Yamaguchi and Yamamoto, 1978], and the name regucalcin was proposed for this Ca^{2+}-binding protein that can regulate the Ca^{2+} or/and calmodulin effects on various enzymes in liver cells [Yamaguchi and Mori, 1988; Yamaguchi, 1992; review]. In recent years, regucalcin has been demonstrated to play multifunctional role as a regulatory protein in intracellular signaling pathway in many cell types [Yamaguchi, 2000a,b, 2005; review].

The rat and human regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. The gene of

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Received 26 June 2006; Accepted 21 July 2006
DOI 10.1002/jcb.21105

regucalcin is highly conserved in vertebrate species [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. Regucalcin are greatly present in liver and kidney cortex, although it is found in other tissues [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogi, 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, NF1-A1, and RGPR-p117 (a novel protein which is related to the regucalcin gene promoter region) have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1998, 1999; Misawa and Yamaguchi, 2001, 2002; Sawada and Yamaguchi, 2005, 2006].

Regucalcin has been proposed to play a physiologic role in maintaining homeostasis and functions as a regulatory protein on intracellular signaling process in many cell types [Yamaguchi, 2000a,b, 2005; reviews]. Regucalcin plays an important role in the maintenance of intracellular Ca^{2+} homeostasis, the inhibitory regulation for various Ca^{2+}-dependent signaling enzyme activities (including protein kinases, thyrosine kinases, protein phosphatases, and NO synthase), protein synthesis, nuclear DNA, and RNA syntheses in cells [Yamaguchi, 2005, review; Tsurusaki and Yamaguchi, 2002a,b; Izumi et al., 2003]. 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; Yamaguchi, 2000b; review].

Overexpression of regucalcin has a suppressive effect on cell proliferation and various signaling factors induced apoptotic cell death in the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002; Izumi and Yamaguchi, 2004a,b; Yamaguchi and Daimon, 2005]. Regucalcin plays a physiologic role in maintaining the homeostasis of cellular response for cell stimulation [Yamaguchi, 2005]. In addition, regucalcin transgenic rats have been shown to induce bone loss and hyperglycemia [Yamaguchi et al., 2002, 2004], suggesting its pathophysiologic role.

Regucalcin with relatively higher levels is also found in kidney cortex [Yamaguchi and Isogi, 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 [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 various Ca^{2+}-dependent enzyme activities [Kurota and Yamaguchi, 1997c; Morooka and Yamaguchi, 2002; Ma and Yamaguchi, 2003] in rat kidney cortex cells. Regucalcin may play a role in the regulation of Ca^{2+}-dependent kidney cell function.

More recently, regucalcin has been shown to express in the cloned normal rat kidney proximal tubular epithelial NRK52E cells, and its mRNA expression has been demonstrated to stimulate by parathyroid hormone or aldosterone, which targets kidney proximal tubular epithelial cells [Nakagawa and Yamaguchi, 2005a]. Overexpression of regucalcin has been shown to suppress cell proliferation [Nakagawa et al., 2005] and apoptotic cell death [Nakagawa and Yamaguchi, 2005b], which are mediated through various intracellular signaling factors, in kidney NRK52E cells.

This study was undertaken to determine whether regucalcin suppresses cell responses that are mediated through intracellular signaling pathways of TNF-α or TGF-β1 in the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin.

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), TNF-α, and TGF-β1 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 *Pst*I site downstream of the translational stop codon, and *Pst*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 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).

Assay of NO Synthase

NO synthase activity in the 5,500*g* supernatant of NRK52E cell homogenate was estimated by the procedure of Lee and Stull [1998] with a minor modification. The enzyme activity was measured for 60 min at 37°C in a reaction mixture (1.0 ml) containing 100 mM HEPES, pH 7.2, 4 mM β-NADPH, 2 mM L-arginine, and the cell protein (60–120 μg/ml) in the absence or presence of both calcium chloride (10 μM) and calmodulin (2.5 or 5.0 μg/ml). The enzyme reaction was terminated by the addition of 1.0 ml of cold 10% trichloroacetic acid and centrifuged to precipitate protein. Produced citrullin in the supernatant was quantified by the method of Boyde and Rahmatullah [1980]. Results were expressed as nanomoles of cirtrullin produced per minute (min) per milligram (mg) of cell protein. Protein concentration was determined by the method of Lowry et al. [1951] using bovine serum albumin as the standard.

Western Blot Analysis

The cells cultured were polled and scraped into 0.5 ml of ice-cold 0.25 M sucrose, and disrupted for 30 s with an ultrasonic device. Scrapped cells were also homogenized in Potter–Elvehjem homogenizer with a teflon pestle. Aliquots of protein (20 μg) of homogenate were mixed with 5× Laemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 10%

polyacrylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 150 mA for 2.5 h [Wessendolf et al., 1993]. The membranes were incubated with a mouse monoclonal human anti-α-smooth muscle actin antibody (DakoCytomation, Glostrup, Denmark) [Fan et al., 1999], which was diluted 1:1,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% (w/v) Tween-20 (washing buffer), and 1% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horseradish peroxidase-linked anti-mouse IgG, which was diluted 1:3,000 with washing buffer containing 1% (w/v) skim milk and again they were washed. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacturer's instruction. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

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 pelletes 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 amplication Smad 2 cDNA were: 5′-GCTCAAGGCGATCGAGAACT-3′ (sense strand, positions 562–581 of cDNA sequence) and 5′-TCCAGGTGGTGGTGTTTCTG-3′ (antisense strand, positions 808–827) [Zhang et al., 1999]. The pair of oligonucleotide primers was designed to amplify a 266-bp sequence from the mRNA of rat Smad 2. Primers for Smad 3 cDNA were: 5′-AGGAGCTGTGCCCTCAGAAG-3′ (sense strand, positions 2819–2839 of cDNA sequence) and 5′-AAGCAGCAGTCCACAGACCA-3′ (antisense strand, positions 3028–3047) [Chen et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 229-bp sequence from the mRNA of rat Smad 3. Primers for NF-κB (p105) cDNA were: 5′-GACCCAAGGACATGGTGGTT-3′ (sense strand, positions 377–396 of cDNA sequence) and 5′-TGTCTGTGAGTTGCCGGTCT-3′ (antisense strand, positions 565–564) [Hammar et al., 2005]. Glyceroaldehyde-3-phosphate 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 μg of total RNAs, supplied RT-PCR 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 35 cycles under the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 60 s at 68°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 or TGF-β1 on Cell Death in Kidney NRK52E Cells Overexpressing Regucalcin

The cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-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 to

obtain subconfluent monolayers, 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 TNF-α or TGF-β1, and further cultured for 72 h. The expression of regucalcin was remarkable in transfectants (data not shown), as shown previously [Nakagawa et al., 2005].

NRK52E cells with subconfluency were cultured for 24, 48, or 72 h in medium without BS containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-β1 (1.0 or 5.0 ng/ml of medium), which is effective concentration. The number of wild-type cells was significantly decreased in culture with TNF-α or TGF-β1. These decreases were significantly prevented in transfectants overexpressing regucalcin (Fig. 1). Culture with TNF-α (10 ng/ml) for 24 h caused a significant decrease in cell number, and this decrease was seen in transfectants (data not shown). The adherent cells were lysed, and then the lysate was separated by electrophoresis in agarose gel. Culture with TNF-α (1.0 ng/ml) or TGF-β1 (5.0 ng/ml) caused DNA fragmentation in wild-type cells (Fig. 2). The effect of those factors on DNA fragmentation was significantly prevented in transfectants. These results indicate that overexpression of regucalcin has a suppressive effect on apoptotic cell death.

NRK52E cells (wild-type) or transfectants with subconfluency were cultured for 72 h in medium without BS containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-β1 (1.0 or 5.0 ng/ml of medium) in the presence or absence of caspase-3 inhibitor (10^{-8} M). TNF-α (Fig. 3)- or TGF-β1 (Fig. 4)-induced decrease in the number of wild-type cells was not seen in the presence of caspase-3 inhibitor. The number of transfectants was not significantly changed in the presence of TNF-α or TGF-β1 with or without caspase-3 inhibitor.

Effect of TNF-α or TGF-β1 on NO Synthase Activity in Kidney NRK52E Cells Overexpressing Regucalcin

NRK52E cells (wild-type) or transfectants with subconfluency were cultured for 24, 48, or 72 h in medium without BS containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-β1 (1.0 or 5.0 ng/ml of medium). NO synthase activity in the wild-type cells was significantly increased by addition of calcium chloride (10 μM) and calmodulin (2.5 or 5.0 μg/ml) into the enzyme reaction mixture (Fig. 5).

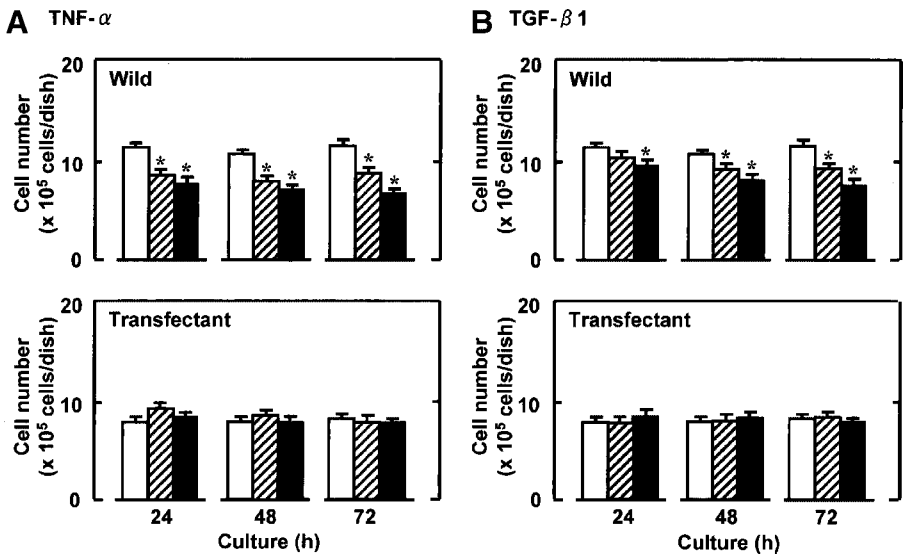

Fig. 1. Effect of TNF-α or TGF-β1 on the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-β1 (1.0 or 5.0 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 (none) value obtained from Figure **A** or **B**. White bars, control (none); hatched bars, TNF-α (0.1 ng/ml) or TGF-β1 (1.0 ng/ml); black bars, TNF-α (1.0 ng/ml) or TGF-β1 (5.0 ng/ml).

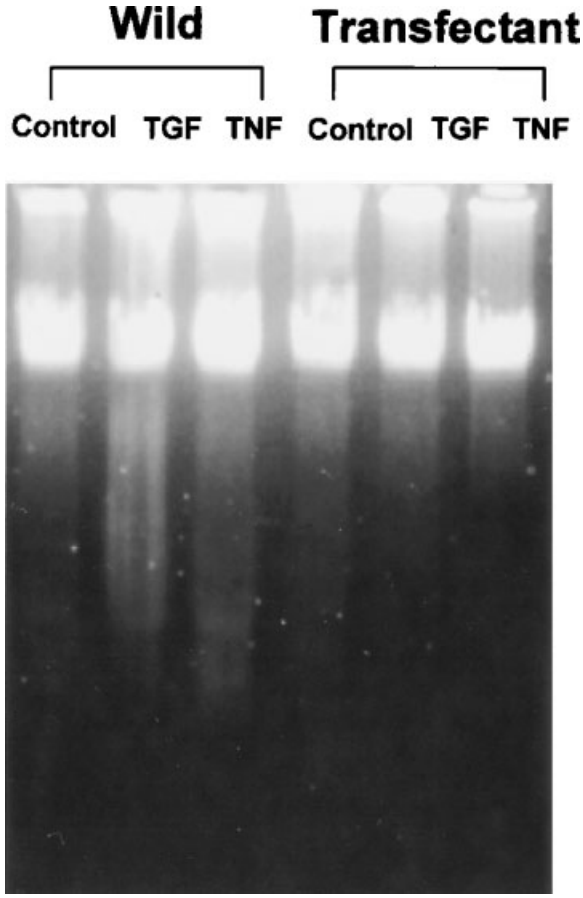

Fig. 2. Effect of TNF-α or TGF-β1 on DNA fragmentation in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchange to culture medium without BS containing either vehicle, TNF-α (1.0 ng/ml of medium), or TGF-β1 (5.0 ng/ml of medium). After medium change, cells were incubated for 72 h, and the lysate (containing 1.0 μg of DNA) of adherent cells were applied to agarose gel. The figure shows one of four experiments with separate cells.

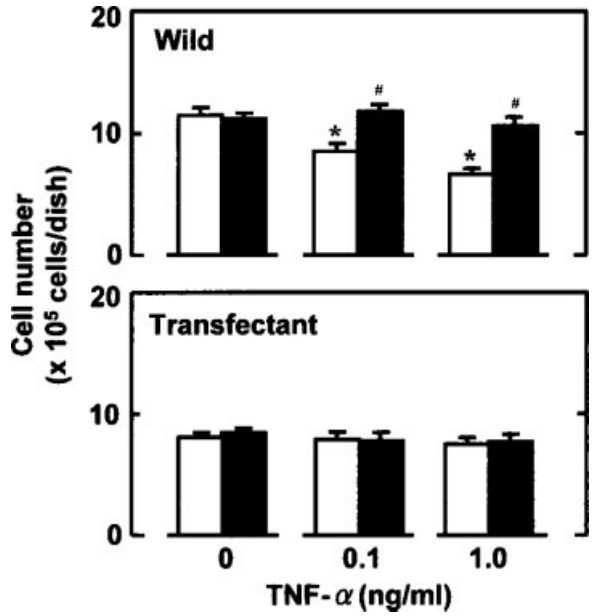

Fig. 3. Effect of caspase inhibitor on TNF-α induced decrease in the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchange to culture medium without BS containing either vehicle or TNF-α (1.0 ng/ml of medium) in the presence or absence of caspase inhibitor (10^{-8} M). After medium change, cells were incubated for 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. $^{\#}P < 0.01$ compared with the value of TNF-α alone. White bars, without caspase inhibitor; black bars, with caspase inhibitor.

The effect of calcium and calmodulin in increasing NO synthase activity was not seen in transfectants (Fig. 5). NO synthase activity in the wild-type cells was significantly increased in culture with TNF-α (1.0 ng/ml) for 48 or 72 h with or without calcium (10 μM) and calmodulin (2.5 μg/ml) in the enzyme reaction mixture (Fig. 6). These increase were not seen in transfectants. Culture with TGF-β1 (1.0 or 5.0 ng/ml) did not cause a significant increase in NO synthase activity in the wild-type cells or transfectants with or without the addition of calcium (10 μM) and calmodulin (2.5 μg/ml) in the enzyme reaction mixture (Fig. 7).

Change in α-Smooth Muscle Actin Level in Kidney NRK52E Cells Overexpressing Regucalcin

The effect of TNF-α or TGF-β1 on α-smooth muscle actin levels in wild-type cells and transfectants was examined (Fig. 8). Cells with subconfluency were cultured for 24 or 72 h in medium without BS containing either vehicle, TNF-α (1.0 ng/ml of medium), or TGF-β1 (5.0 ng/ml). α-Smooth muscle actin level in the cell lysate was estimated using Western blot analysis. Culture with TNF-α or TGF-β1 caused a remarkable increase in α-smooth muscle actin levels in wild-type cells cultured for 24 or 72 h. This increase was significantly prevented in transfectants.

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

The change in mRNA expression of TNF-α- or TGF-β-related signal proteins in the cloned

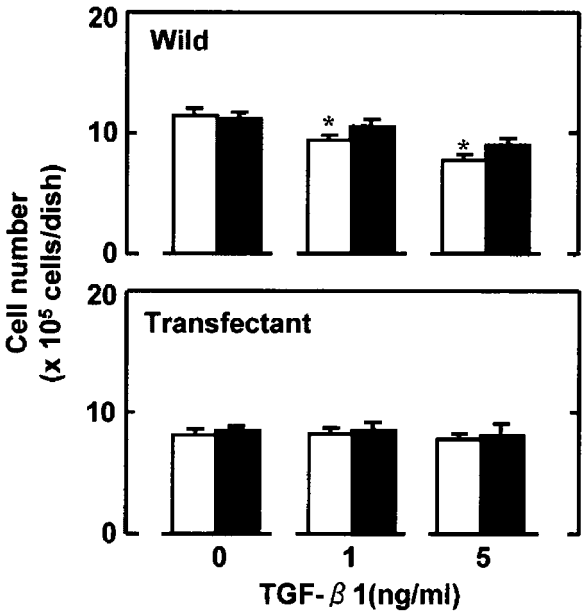

Fig. 4. Effect of caspase inhibitor on TGF-β1-induced decrease in the number of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchange to culture medium without BS containing either vehicle or TGF-β1 (5.0 ng/ml of medium) in the presence or absence of caspase inhibitor (10^{-8} M). After medium change, cells were incubated for 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, without caspase inhibitor; black bars, with caspase inhibitor.

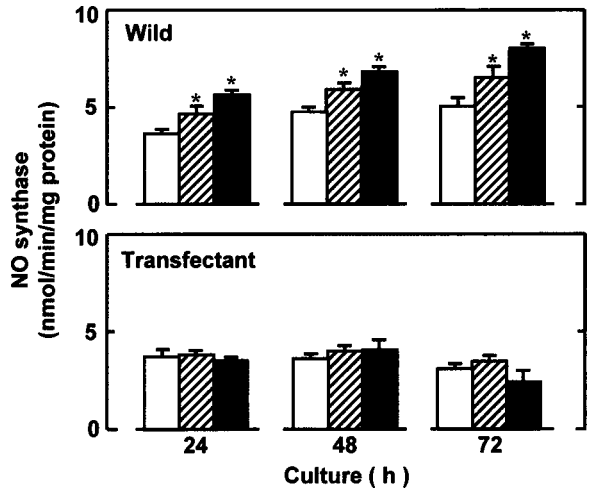

Fig. 5. Effect of calcium and calmodulin on NO synthase activity in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells were subconfluency were exchanged to culture medium without BS. After medium change, cells were incubated for 24, 48, or 72 h and NO synthase activity in cell lysate was measured. Enzyme reaction mixture contained either vehicle or calcium chloride (10 μM) plus calmodulin (2.5 or 5.0 μg/ml). Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$ compared with the control (none) value. White bars, none; hatched bars, calcium (10 μM) plus calmodulin (2.5 μg/ml); black bars, calcium (10 μM) plus calmodulin (5.0 μg/ml).

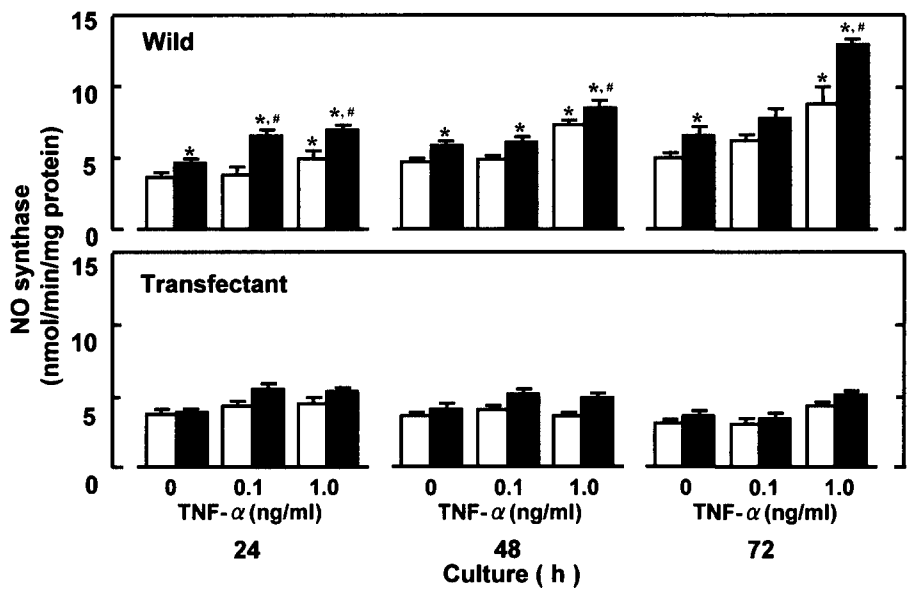

Fig. 6. Effect of TNF-α on NO synthase activity in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchanged to culture medium without BS containing either vehicle or TNF-α (0.1 or 1.0 ng/ml of medium). After medium change, cells were incubated for 24, 48, or 72 h and NO synthase activity was measured in reaction mixture containing either vehicle or calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$ compared with the control value without TNF-α. $^{\#}P < 0.01$ compared with the value without addition of calcium and calmodulin. White bars, without calcium and calmodulin; black bars, with calcium and calmodulin.

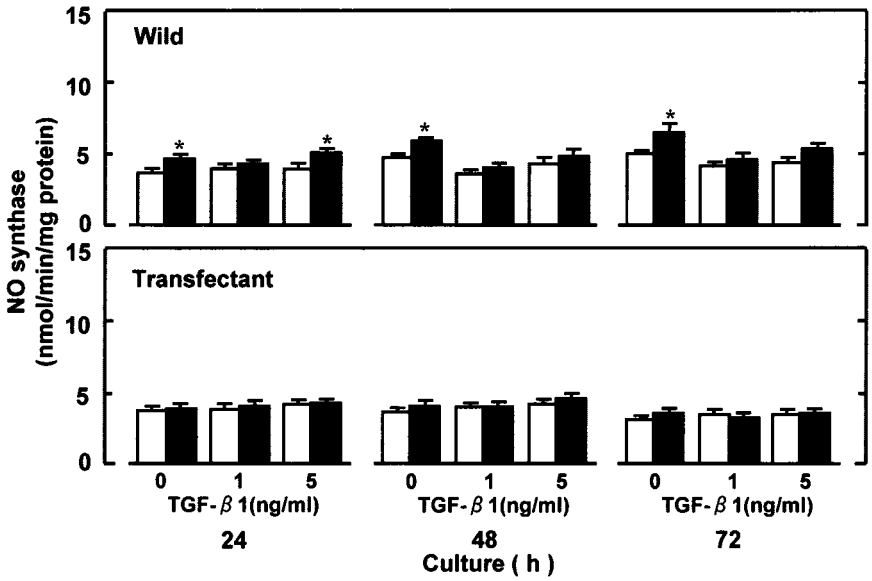

Fig. 7. Effect of TGF-β1 on NO synthase activity in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchanged to culture medium without BS containing either vehicle or TGF-β1 (1.0 or 5.0 ng/ml of medium). After medium change, cells were incubated for 24, 48, or 72 h and NO synthase activity was measured in reaction mixture contained either vehicle or calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of six experiments. $*P < 0.01$ compared with the control value without TGF-β1. White bars, without calcium and calmodulin; black bars, with calcium and calmodulin.

normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells with subconfluency was examined. Cells were cultured for 72 h in medium without BS. The expression of Smad 2 or NF-κB mRNA was significantly increased in transfectants as compared with that of wild-type cells (Fig. 9). Smad 3 or G3PDH mRNA expression was not significantly changed in transfectants.

The expression of NF-κB mRNA in wild-type cells was significantly increased with culture for 72 h in the presence of TNF-α (1.0 ng/ml of medium) (Fig. 10). The effect of TNF-α in increasing NF-κB mRNA expression was not further enhanced in transfectants.

Culture with TGF-β1 (5.0 ng/ml) for 72 h caused a significant increase in Smad 2 mRNA expression in wild-type cells, while it did not have a significant effect on Smad 3 mRNA expression (Fig. 11). TGF-β1 did not significantly enhanced Smad 2 mRNA expression in transfectants (Fig. 11).

DISCUSSION

Regucalcin plays a multifunctional role as a regulatory protein of intracellular signaling pathway in many cell types [Yamaguchi, 2005; review]. Overexpression of regucalcin has a suppressive effect on cell proliferation and apoptotic cell death induced by signaling factors in the cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro [Nakagawa et al., 2005; Nakagawa and Yamaguchi, 2005b]. Moreover, this study was undertaken to determine whether overexpression of regucalcin suppresses cell responses that are mediated through signaling process following stimulation with TNF-α or TGF-β1 in NRK52E cells. We found that overexpression of regucalcin suppresses various cellular responses for TNF-α or TGF-β1 in NRK52E cells.

Culture with TNF-α or TGF-β1 was found to induce cell death in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cell death induced by TNF-α or TGF-β1 was significantly prevented with culture of caspase-3 inhibitor, suggesting that the apoptosis is partly mediated through mechanism, which is involved in caspase-3 in NRK52E cells. Overexpression of regucalcin had a suppressive effect on apoptotic cell death induced by TNF-α or TGF-β1. Regucalcin may influence on the mechanism of cell death that is mediated through caspase-3 in NRK52E cells. Regucalcin

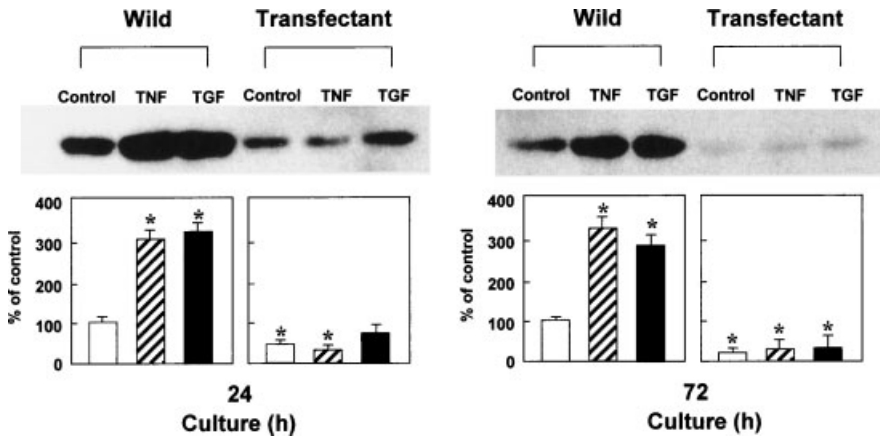

Fig. 8. Change in α-smooth muscle actin level in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchanged to culture medium without BS containing either vehicle, TNF-α (1.0 ng/nl of medium), or TGF-β1 (5.0 ng/ml). After medium change, cells were incubated for 24 or 72 h, and actin level in the cell lysate was measured using Western blot analysis. The densitometric data for each α-smooth muscle actin levels were indicated as % (mean ± SEM of four experiments with separate samples) of the control obtained from wild-type cells cultured for 24 or 72 h, respectively. $^*P < 0.01$ compared with the control value.

has been shown to localize in the nucleus of NRK52E cells [Nakagawa and Yamaguchi, 2006]. It is speculated that regucalcin inhibits nuclear DNA fragmentation that is partly related to caspase-3. Regucalcin has been demonstrated to have suppressive effect on cell death induced by TNF-α in NRK52E cells [Nakagawa and Yamaguchi, 2005b]. This study demonstrates, moreover, that regucalcin has a suppressive effect on cell death, which is mediated through signaling mechanism of TGF-β1 in NRK52E cells.

Addition of calcium and calmodulin in the enzyme reaction mixture caused a significant increase in NO synthase activity in the homogenate obtained from NRK52E cells. The effect of calcium and calmodulin in increasing NO synthase activity was not seen in the homogenate obtained from transfectants overexpressing regucalcin. Regucalcin has been shown to bind to calmodulin and to have an inhibitory effect on calcium and calmodulin-dependent enzyme activation [Omura and Yamaguchi, 1998]. NO synthase activity was significantly increased in wild-type cells cultured with TNF-α. Such an increase was not seen in culture with TGF-β1. Overexpression of regucalcin was found to have a suppressive effect on the

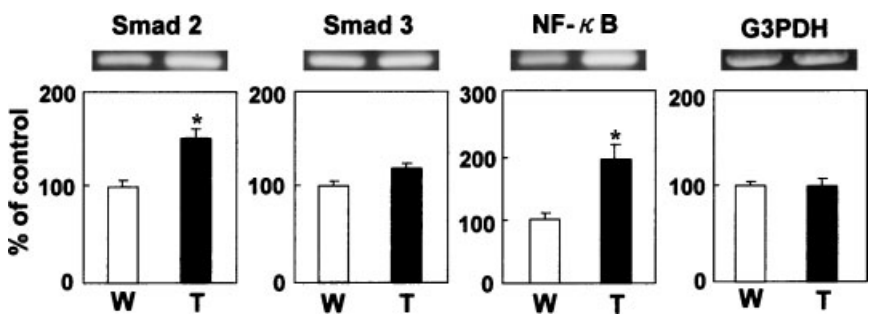

Fig. 9. Change in mRNA expression of proteins which are related to signaling of TNF-α or TGF-β1 in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type; W) or regucalcin (RC)/pCXN2-transfected cell (T). Cells with subconfluency were exchanged to culture medium without BS. After medium change, cells were cultured for 72 h. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The densitometric data for each mRNA levels were indicated as % control (mean ± SEM of five experiments). $^*P < 0.01$ compared with the control value.

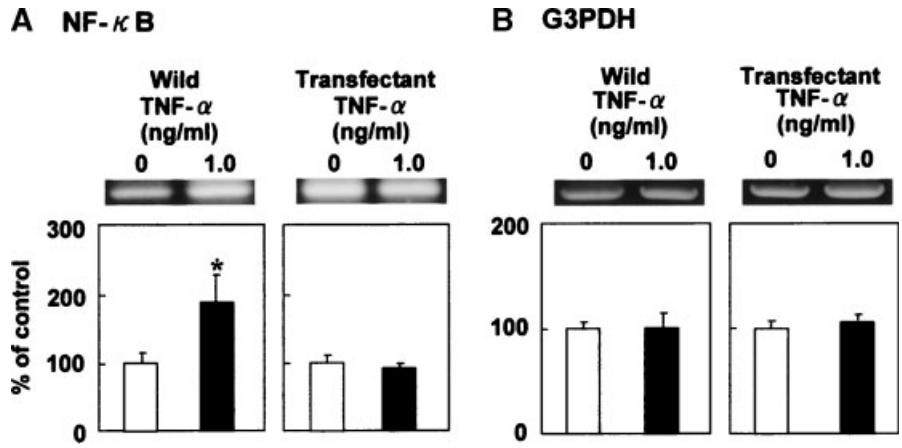

Fig. 10. Effect of TNF-α on NF-κB mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchanged to culture medium without BS containing either vehicle or TNF-α (1.0 ng/ml of medium). After medium change, cells were cultured for 72 h. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The densitometric data for each mRNA levels were incubated as % of control (mean ± SEM of five experiments). **A**, NF-κB mRNA; **B**, G3PDH mRNA. $^*P < 0.01$ compared with the control value.

elevation of NO synthase activity in NRK52E cells cultured with TNF-α. The suppressive effect of regucalcin on TNF-α-induced cell death may partly be involved in its inhibitory effect on NO synthase activity in NRK52E cells.

The change in α-smooth muscle actin level in NRK52E cells (wild type) cultured with TNF-α or TGF-β1 was examined using Western blot analysis. Culture with TNF-α or TGF-β1 caused a remarkable increase in α-smooth muscle actin level in wild-type cells. Such an increase was only slight in transfectants. In addition, the expression of α-smooth muscle actin was markedly suppressed in transfectants cultured without TNF-α or TGF-β1. These findings demonstrate that overexpression of regucalcin has a suppressive effect on the expression of α-smooth muscle actin in NRK52E cells cultured with TNF-α or TGF-β1. It is speculated that regucalcin regulates signaling pathway which is mediated through TNF-α or TGF-β1 to stimulate the expression of α-smooth muscle actin in NRK52E cells. TGF-β1 is a key mediator that regulates transdifferentiation of NRK52E cells into

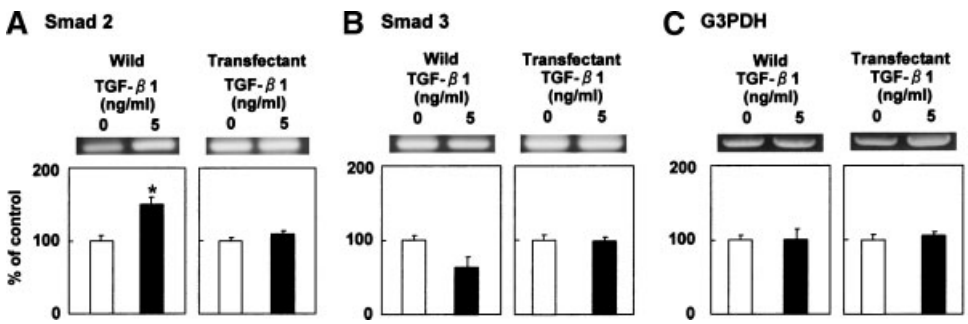

Fig. 11. Effect of TGF-β1 on Smad 2 or Smad 3 mRNAs expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild type) or regucalcin (RC)/pCXN2-transfected cells. Cells with subconfluency were exchanged to culture medium without BS containing either vehicle or TGF-β1 (5.0 ng/ml of medium). After medium change, cells were cultured for 72 h. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The densitometric data for each mRNA levels were incubated as % of control (mean ± SEM of five experiments). **A**, Smad 2 mRNA; **B**, Smad 3 mRNA; **C**, G3PDH mRNA. $^*P < 0.01$ compared with the control value.

myofibroblasts expressing α-smooth muscle actin [Fan et al., 1999]. This may contribute to renal fibrosis associated with overexpression of TGF-β1 within the diseased kidney [Fan et al., 1999]. Whether regucalcin regulates transdifferentiation to renal fibrosis in NRK52E cells with TGF-β1 or TNF-α remains to be elucidated.

Overexpression of regucalcin caused a remarkable increase in the expression of mRNA of Smad 2, which is involved in signal transduction of TGF-β1 [Zhang et al., 1999], or NF-κB, which is related to signaling of TNF-α [Hammar et al., 2005], in NRK52E cells. Such an increase was not seen in Smad 3 mRNA expression in transfectants. This finding suggests that regucalcin stimulates the gene expression of Smad 2 or NF-κB, which is related to signaling mechanism of TNF-α or TGF-β1. At present, the mechanism by which regucalcin stimulates gene expression of Smad 2 or NF-κB is unknown.

Culture with TNF-α or TGF-β1 caused a remarkable increase in the expression of mRNAs of NF-κB or Smad 2 in NRK52E cells (wild type). TNF-α or TGF-β1 did not have stimulatory effects on the expression of NF-κB or Smad 2 mRNAs in transfectants overexpressing regucalcin. This mechanism is unknown at present. Regucalcin may have suppressive effect on signaling pathway which TNF-α or TGF-β1 stimulates gene expression of NF-κB or Smad 2 in NRK52E cells. These cytokines may not have enhancing effects on gene expression of NF-κB or Smad 2 in transfectants. In addition, it is possible that regucalcin stimulates gene expression of NF-κB or Smad 2 through the mechanisms, which differ from signaling pathway of TNF-α or TGF-β1. Presumably, the suppressive effects of regucalcin on apoptotic cell death and α-smooth muscle actin expression may not involve in the expressions of NF-κB or Smad 2 stimulated by TNF-α or TGF-β1 n NRK52E cells.

In conclusion, it has been demonstrated that overexpression of regucalcin has suppressive effects on cell responses which are mediated through TNF-α or TGF-β1 in the cloned normal rat kidney proximal tubular epithelial NRK52E cells.

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