

The potential role of regucalcin in kidney cell regulation: Involvement in renal failure (Review)

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Abstract. The kidneys play a physiologic role in the regulation of urine formation and nutrient reabsorption in the proximal tubule epithelial cells. Kidney development has been shown to be regulated through calcium (Ca^{2+}) signaling processes that are present through numerous steps of tubulogenesis and nephron induction during embryonic development of the kidneys. Ca^{2+} -binding proteins, such as calbindin-D28k and regucalcin are important proteins that are commonly used as biomarkers in pronephric tubules, and the ureteric bud and metanephric mesenchyme. Previous research on regucalcin focused on Ca^{2+} sensors that are involved in renal organogenesis and the link between Ca^{2+} -dependent signals and polycystins. Moreover, regucalcin has been highlighted to play a multifunctional role in kidney cell regulation. The regucalcin gene, which is localized on the X chromosome, is regulated through various transcription factors. Regucalcin has been found to regulate intracellular Ca^{2+} homeostasis in kidney proximal tubule epithelial cells. Regucalcin has been demonstrated to regulate the activity of various enzymes that are involved in intracellular signaling pathways. It has been noted that regucalcin suppresses DNA synthesis and regulates the gene expression of various proteins related to mineral transport, transcription factors, cell proliferation and apoptosis. The overexpression of regucalcin has been shown to exert suppressive effects on cell proliferation and apoptotic cell death, which are stimulated by various stimulatory factors. Moreover, regucalcin gene expression was found to be involved in various pathophysiological states, including renal failure. This review discusses recent findings concerning the potential role of regucalcin as a regulatory protein in the kidney proximal tubule epithelial cells.

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1. Introduction

The kidneys play a physiologic role in the regulation of urine formation and nutrient reabsorption in the proximal tubule epithelial cells. Kidney development has been shown to be regulated through calcium (Ca^{2+}) signaling processes that are present through numerous steps of tubulogenesis and nephron induction during embryonic development of the kidneys (1). Ca^{2+} -binding proteins, such as calbindin-D28k and regucalcin have been shown to be important proteins that are commonly used as biomarkers in pronephric tubules, and the ureteric bud and metanephric mesenchyme (1). Thus, regucalcin has been focused to be Ca^{2+} sensors that are involved in renal organogenesis in the link between Ca^{2+} -dependent signals and polycystins. Moreover, there is accumulating evidence that regucalcin plays a multifunctional role in kidney cell regulation (2).

Regucalcin, which was discovered in 1978 as a Ca^{2+} -binding protein (3), is known to play a pivotal role as a suppressor protein in intracellular Ca^{2+} signaling in various types of cells and tissues (4-6). The regucalcin gene, which is localized on the X chromosome, is identified in over 15 species consisting of the regucalcin family and is highly conserved in vertebrate species throughout evolution (7). The rat regucalcin gene consists of 7 exons and 6 introns (7). Various transcription factors [including activator protein (AP)-1, nuclear factor I-A1 (NF1-A1), regucalcin gene promoter region-related protein 117 (RGPR-p117), β -catenin, SP1 and others] have been identified as enhancers and suppressors of regucalcin gene expression (7,8). Regucalcin gene expression has been shown to be pronounced in the liver and kidney proximal tubule epithelial cells in rats and is regulated by various transcription factors (1,7,9).

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Regucalcin plays a role in the regulation of Ca^{2+} in kidney proximal tubule epithelial cells. It is involved in the regulation of intracellular Ca^{2+} homeostasis and thus in the transcellular transport and reabsorption of Ca^{2+} from filtrated urinary Ca^{2+} , in the suppression of cell proliferation and apoptotic cell death that are mediated through various signaling factors, and in the regulation of the gene expression of various proteins related to mineral transport-related proteins, transcription factors, cell proliferation and apoptosis-related proteins (4-8). This review discusses the recent findings concerning the potential role of regucalcin in the regulation of the kidney proximal tubule epithelial cells.

2. Various factors regulate regucalcin gene expression

Regucalcin in rat kidney tissues is estimated to be present in the range of $1.74\text{--}3.50 \times 10^{-6}$ moles/g tissues in male or female rats, as measured using an enzyme-linked immunoadsorbent assay (9). This expression does not decrease with aging (9). Regucalcin mRNA expression is predominant in the kidney cortex, but not in the medulla of rats (10). Kidney cortex is comprised of nephrons which include the glomerulus and renal tubule. The transcription factors, NF1-A1 and RGPR-p117, which were identified as hepatic nuclear factors that bind to the TTGGC(N)₆CC sequence of the rat regucalcin gene promoter region, have been shown to enhance regucalcin gene expression (6,7,11). RGPR-p117 was found to be a novel transcription factor (8). The regucalcin gene has been shown to be expressed in kidney proximal tubule epithelial NRK52E cells derived from normal rat kidney cortexes (12). NF1-A1 and RGPR-p117, which are localized in the nuclei of NRK52E cells, have been shown to increase regucalcin promoter activity (13-15). The enhanced regucalcin gene promoter activity with the overexpression of NF1-A1 or RGPR-p117 has been shown to be mediated through protein phosphorylation and dephosphorylation, which are regulated by Ca^{2+} -dependent protein kinases, mitogen-activated protein kinase (MAPK) and protein phosphatases in NRK52E cells (13-15). Thus, NF1-A1 or RGPR-p117 may play an essential role as transcription factors (enhancers) in regucalcin promoter activity in rat kidney cells. The involvement of other transcription factors remains to be elucidated.

Regucalcin gene expression has been shown to be regulated through various hormones. Regucalcin mRNA expression in the kidney cortex was shown to be markedly stimulated after a single intraperitoneal administration of calcium chloride in normal and thyroparathyroidectomized rats with calcitonin and parathyroid hormone (PTH) deficiencies *in vivo* (10), which is known to regulate calcium reabsorption in kidney proximal tubule cells (16,17). The stimulatory effect of calcium administration on regucalcin mRNA expression in the kidneys was blocked by treatment with trifluoperazine (TFP), an inhibitor of Ca^{2+} /calmodulin, suggesting that its expression is mediated through Ca^{2+} /calmodulin, which is involved in the activation of protein kinases (10,18). PTH, 1,25-dihydroxyvitamin D₃ and calcitonin play a role in the regulation of calcium transport in NRK52E cells (16,17). Among these hormones, PTH was found to stimulate regucalcin mRNA expression and its protein levels in NRK52E cells (12) *in vitro*. PTH exerts a stimulatory effect on the reabsorption of calcium in the kidney proximal

tubule (16,17). The effects of PTH are known to be mediated by cyclic AMP (cAMP) or inositol 1,4,5-trisphosphate (IP₃)-released Ca^{2+} and protein kinase C in cells (19). In a previous study, regucalcin mRNA expression was enhanced by dibutyryl cAMP or phorbol-12-myristate 13-acetate (PMA), an activator of protein kinase C, in NRK52E cells (12), suggesting that it is partly mediated through signaling pathways related to cAMP or protein kinase C in NRK52E cells.

PD98059 is an inhibitor of the extracellular signal-related kinase (ERK) pathway (20). Regucalcin mRNA expression in NRK52E cells is not altered in the presence of PD98059 *in vitro* (12), suggesting that its expression is not mediated through a MAPK that is related to the ERK pathway. Regucalcin mRNA expression has been shown to be suppressed following culture with staurosporine, an inhibitor of protein kinase C in NRK52E cells, supporting the hypothesis that its expression is mediated through a cell signaling pathway related to protein kinase C in kidney cells (12). Regucalcin mRNA expression is not altered by culture with vanadate, which is an inhibitor of protein tyrosine phosphatase (21), in NRK52E cells (12). Of note, regucalcin mRNA expression has been found to be suppressed following culture with tumor necrosis factor- α (TNF- α) or transforming growth factor- β (TGF- β) in NRK52E cells (22). The effects of TNF- α are mediated through the nuclear factor- κ B (NF- κ B) signaling pathways. TGF- β is mediated through the Smads signaling pathways. Regucalcin mRNA expression may be suppressed through transcription factors that are related to NF- κ B and Smads.

Steroid hormones have been shown to regulate regucalcin gene expression in kidney cells. Regucalcin mRNA expression was shown to be enhanced following culture with dexamethasone in NRK52E cells *in vitro* (12). Regucalcin mRNA expression was shown to be stimulated after a single subcutaneous administration of dexamethasone in rats, whereas it was suppressed after a single subcutaneous administration of aldosterone or estrogen *in vivo* (23). The administration of hydrocortisone in rats did not alter regucalcin mRNA expression in the kidney cortex *in vivo* (23). It has been suggested that the regucalcin gene promoter region is the location of the response elements for glucocorticoid, aldosterone or estrogen receptors. Regucalcin mRNA expression has been shown to be suppressed in the kidney cortex of adrenalectomized (ADX) rats, which diminishes the secretion of endogenous steroid hormones from adrenal glands *in vivo* (24,25). Thus, the adrenal glands may participate in the regulation of regucalcin mRNA expression in the kidney cortex of rats.

3. Regucalcin regulates intracellular calcium transport

The kidneys play a physiological role in the regulation of Ca^{2+} homeostasis in the blood through the reabsorption of urinary Ca^{2+} (24,25). Renal cortex cells, which constitute the proximal tubule epithelial cells, may play a role in the reabsorption of urinary Ca^{2+} . Active Ca^{2+} reabsorption involves transcellularly transportation, and Ca^{2+} pumps in the basolateral membranes are involved to overcome the energy barriers at the peritubular cell side (24,25). The regulation of intracellular Ca^{2+} homeostasis is important in the promotion of intracellular Ca^{2+} transport. Intracellular Ca^{2+} homeostasis is regulated through the plasma membrane (Ca^{2+} -Mg²⁺)-adenosine 5'-triphospha-

tase (ATPase), microsomal Ca^{2+} -ATPase, mitochondrial Ca^{2+} uptake and nuclear Ca^{2+} transport in the cells. The Ca^{2+} -ATPase system exceeds the capacity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and plays a primary role in Ca^{2+} homeostasis of in rat kidney cortex cells (24). Regucalcin has been found to play a role as an activator of the adenosine-5'-triphosphate (ATP)-dependent Ca^{2+} pumps (Ca^{2+} -ATPase) in the basolateral membranes isolated from the rat kidney cortex; regucalcin (100 and 1,000 nM) increased Ca^{2+} -ATPase activity and stimulated $^{45}\text{Ca}^{2+}$ uptake by the basolateral membranes *in vitro* (26). The effect of regucalcin on Ca^{2+} pump enzyme activity was inhibited in the presence of vanadate, an inhibitor of phosphorylation of ATPase, *N*-ethylmaleimide (SH-group modifying reagent) and DcAMP, but not IP_3 (26). It was suggested that regucalcin binds to the lipids at the site of the Ca^{2+} pump enzyme in the basolateral membranes of the rat kidney cortex and may activate the enzyme by acting on the SH-group of the enzyme (26).

Regucalcin has been shown to increase Ca^{2+} -ATPase activity and ATP-dependent calcium uptake in the microsomes isolated from the rat kidney cortex *in vitro* (27). This increase resulted from the binding of regucalcin to the SH-group of active sites of the enzyme and the stimulation of the phosphorylation of the enzyme in the microsomes of the rat kidney cortex (27). Kidney cortex microsomal Ca^{2+} -ATPase activity and ATP-dependent Ca^{2+} uptake were inhibited by DcAMP or IP_3 (27). Calmodulin increased microsomal Ca^{2+} -ATPase activity, and this increase was lower than that effected by regucalcin; both proteins may be important as activators in the microsomal ATP-dependent Ca^{2+} sequestration (27).

An ATP-dependent Ca^{2+} uptake system (Ca^{2+} uniporter) exists in the mitochondria of the kidney cortex of rats (28). Regucalcin has also been shown to stimulate Ca^{2+} -ATPase-related Ca^{2+} uniporter activity in the mitochondria (28). This effect was completely blocked by ruthenium red or lanthanum chloride, which is a specific inhibitor of Ca^{2+} uniporter in the mitochondria (28), suggesting that regucalcin stimulates Ca^{2+} -ATPase-related Ca^{2+} uniporter activity in renal cortex mitochondria. Regucalcin has been suggested to bind to the membranous lipids of renal cortex mitochondria and act on the SH-groups, which are active sites of Ca^{2+} -ATPase (28).

It has been observed that reabsorption of urinary calcium is promoted through transcellular Ca^{2+} transport in renal proximal tubule epithelial cells (24,25). Thus, regucalcin may play a physiological role in the regulation of intracellular Ca^{2+} homeostasis in the renal proximal tubular epithelial cells, due to its activation of ATP-dependent Ca^{2+} -transport systems in the basolateral membranes, microsomes and mitochondria. Regucalcin may promote Ca^{2+} reabsorption, which is based on the ATP-dependent transcellular transport of Ca^{2+} , in the proximal tubular epithelial cells of the nephron tubule of the kidney cortex. Thus, regucalcin may play a physiological role in the regulation of Ca^{2+} homeostasis in the blood through reabsorption of urinary Ca^{2+} in the kidneys.

Regucalcin has been demonstrated to regulate the gene expression of various proteins related to mineral ion transport in kidney cells. To determine the role of endogenous regucalcin, NRK52E cells (transfectants) overexpressing regucalcin were generated in a previous study (29). In regucalcin/pCXN2-transfected cells regucalcin overexpression was increased 21-fold as compared with that of the parental wild-type NRK52E

cells (29). The overexpression of endogenous regucalcin was found to increase rat outer medullary K^+ channel (ROMK) mRNA expression in NRK52E cells, although it did not alter the expression of type II NaPi cotransporter (NaPi-IIa), Na^+ , K^+ -ATPase, epithelial sodium channel (ENaC) or angiotensinogen mRNAs (30). Culture with aldosterone caused an increase in ENaC, Na^+ , K^+ -ATPase and ROMK mRNA expression in wild-type NRK52E cells (30). The effect of aldosterone in increasing ENaC and Na^+ , K^+ -ATPase mRNA expression was not observed in the transfectants (30). Aldosterone was shown to upregulate regucalcin mRNA expression in NRK52E cells (12). The stimulatory effect of aldosterone on ENaC and Na^+ , K^+ -ATPase mRNA expressions may thus be partly mediated through endogenous regucalcin in NRK52E cells.

The overexpression of endogenous regucalcin was found to exert suppressive effects on the mRNA expression of the L-type Ca^{2+} channel and calcium-sensing receptor (CaR), which regulate intracellular Ca^{2+} signaling and renal Ca^{2+} transport in NRK52E cells (30,31). The entry of Ca^{2+} through L-type Ca^{2+} channels induces mitochondrial disruption and cell death (31). The blockade of Ca^{2+} influx through L-type Ca^{2+} channels has been shown to attenuate mitochondrial injury and apoptosis in hypoxia of renal tubular cells (31). Regucalcin may regulate the intracellular Ca^{2+} signaling pathway, which is mediated through its suppressive effect on L-type Ca^{2+} channel or CaR mRNA expressions in the kidney proximal tubular epithelial cells.

Taking the above discussion into account, it is suggested that regucalcin plays a pivotal role in the regulation of intracellular Ca^{2+} homeostasis and mineral transport in kidney proximal tubule epithelial cells, as summarized in Fig. 1.

4. Regucalcin regulates cell signaling-related enzyme activity

Regucalcin has been demonstrated to exert suppressive effects on the activity of various enzymes that regulate cell-signaling pathways in kidney cells. Multifunctional Ca^{2+} /calmodulin-dependent protein kinases play an important role in the Ca^{2+} signaling response of many cells (32). Regucalcin (10-1,000 nM) was found to exert suppressive effects on the activation of Ca^{2+} /calmodulin-dependent protein kinase in the cytosol of rat kidney cortex *in vitro* (33). This effect was also noted when higher concentrations of calcium chloride (100-1,000 μM) and calmodulin (4-20 $\mu\text{g/ml}$) were added to the enzyme reaction mixture (33), suggesting that regucalcin has a direct inhibitory effect on the protein kinase in renal cortex cytosol.

Protein kinase C is a diacylglycerol-activated Ca^{2+} - and phospholipid-dependent protein kinase that is related to Ca^{2+} signaling in many cell types. Regucalcin has been found to inhibit protein kinase C activity in the cytosol of rat kidney cortex *in vitro* (34). The inhibitory effect of regucalcin on protein kinase C activity was not observed in a reaction mixture without Ca^{2+} , but was noted in the presence of Ca^{2+} , phosphatidylserine and dioctanoylglycerol (34). Moreover, regucalcin suppressed protein kinase C activity caused by the addition of diacylglycerol or PMA, which directly activated the enzyme, in the presence of both Ca^{2+} and phosphatidylserine (34). Thus, it is suggested that regucalcin directly binds to protein kinase C.

Protein phosphorylation-dephosphorylation is a universal mechanism through which numerous cellular events are regulated (21). Protein phosphatases play an important role

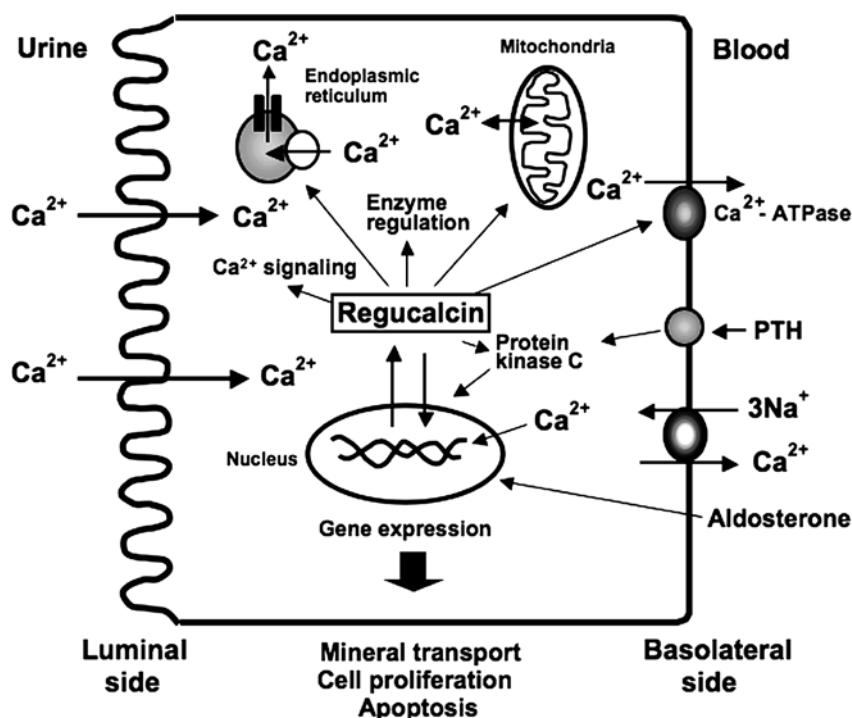


Figure 1. Regucalcin plays a multifunctional role in the kidney proximal tubule epithelial cells. Regucalcin gene expression is stimulated through Ca^{2+} signaling, parathyroid hormone (PTH) or aldosterone, which regulates mineral transportation in the cells. Regucalcin stimulates transcellular transportation of reabsorbed urinary Ca^{2+} . Regucalcin suppresses the activation of various enzymes, which are related to Ca^{2+} signaling pathways. Regucalcin suppresses cell proliferation and apoptosis, which that is stimulated by various factors. Nuclear localization of regucalcin is enhanced by the protein kinase C-related signaling process. Nuclear regucalcin suppresses DNA synthesis and regulates the gene expression of many proteins that mediate mineral transport, cell proliferation and apoptotic cell death. Thus, regucalcin plays a potential role in kidney cell regulation. ATP, adenosine 5'-triphosphatase.

in intracellular signal transduction due to hormonal stimulation. Calcineurin, a calmodulin-binding protein, possesses a Ca^{2+} -dependent and calmodulin-stimulated protein phosphatase that is a protein serine/threonine phosphatase (35). Regucalcin was found to inhibit calcineurin activity in rat renal cortex cytosol *in vitro* (36). This inhibitory effect was independent of Ca^{2+} , suggesting that regucalcin acted directly on the enzyme (36). Regucalcin has been shown to bind to calmodulin in a study using calmodulin-agarose beads *in vitro* (37). The inhibitory effect of regucalcin on enzyme activity may be related to its binding to calmodulin. Regucalcin has also been shown to suppress the activities of Ca^{2+} /calmodulin-independent protein phosphatases for tyrosine, phosphoserine and phosphothreonine in rat renal cortex cytosol (38). The presence of anti-regucalcin monoclonal antibody in an enzyme reaction mixture caused an increase in protein phosphatase activity toward three phosphoamino acids in the renal cortex cytosol (38).

Endogenous regucalcin may suppress the activity of various protein phosphatases in the kidney cortex cells. Of note, nuclear regucalcin was found to suppress calcineurin, serine/threonine phosphatase and tyrosine phosphatase that are present in the nucleus of rat kidney cortex cells (39). The administration of calcium in rats was shown to increase the levels of regucalcin and cause a corresponding rise in protein phosphatase activity in the cytoplasm and nucleus of the kidney cortex *in vivo* (40). The presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture caused an increase in protein phosphatase in the cytoplasm and nucleus of normal

rat kidney cortex (40). The increased levels of endogenous regucalcin were suggested to suppress protein phosphatase activity enhanced in the cytoplasm and nucleus of the kidney cortex of calcium-administered rats. The dephosphorylation of many phosphorylated proteins is regulated by protein phosphatase, which is implicated in nuclear transcriptional regulation in various cell types (21). Thus, it is posited in this study that regucalcin may play a role in the regulation of nuclear signaling-related gene expression, through the inhibition of nuclear protein phosphatase activity.

cAMP, which is generated through the activation of the plasma membrane adenylate cyclase due to hormonal stimulation, activates the cAMP-dependent protein kinase, which plays an important role in the cAMP signaling pathway. cAMP is degraded by cAMP phosphodiesterase, which is activated by Ca^{2+} /calmodulin (41). Regucalcin was shown to inhibit Ca^{2+} /calmodulin-dependent cAMP phosphodiesterase activity in the cytosol of rat renal cortex (41). Regucalcin plays a role in the regulation of both cAMP-dependent and Ca^{2+} -dependent signaling pathways, which are modulated through hormonal stimulation in the renal cortex cells.

Nitric oxide (NO) acts as a messenger or modulator molecule in many biological systems (42). NO has an unpaired electron that reacts with proteins and targets primarily through their thiol or heme groups, and it acts as a messenger or modulator molecule in many biological systems. NO is produced from L-arginine with L-citrulline as a co-product in a reaction catalysed by NO synthase that requires Ca^{2+} /calmodulin (42). Regucalcin was found to inhibit Ca^{2+} /calmodulin-dependent NO synthase

activity in the renal cortex cytoplasm of rats (43). NO acts as a messenger or modulator in kidney cortex cells. Regucalcin may play a role as a suppressor protein in NO production in the kidney cells and may regulate many cellular events that are involved in NO signaling.

Of note, regucalcin was found to stimulate proteolytic activity in the cytoplasm of rat kidneys (44,45). Regucalcin uniquely activated thiol proteases (including calpines) independently of Ca^{2+} in the cytoplasm of rat kidney cortex, although it did not have an effect on serine proteases and metalloproteases (44,45). Regucalcin was shown to directly act on the SH-groups of protease in the kidney cortex cytoplasm. Regucalcin activated protease at concentrations of 10-250 nM in *in vitro* experiments (44). The concentration of regucalcin in the cytosol of rat kidney tissues may be $\sim 5.3 \mu\text{M}$ (9). Regucalcin may play a physiological role in the activation of thiol proteases in renal cortex cells. Calpains, which are thiol (SH) proteases, are ubiquitous, non-lysosomal and Ca^{2+} -dependent proteases (46). The ability of calpains to alter limited proteolysis, the activity or function of numerous cytoskeletal proteins, protein kinases, receptors and transcription factors is related to Ca^{2+} -regulated cellular functions (45). Regucalcin may play a pivotal role in the degradation of proteins that are involved in the regulation of signaling pathways.

As described above, regucalcin may play a pivotal role as a regulatory protein involved in the activation of many enzymes that are related to signaling pathways, which are mediated through Ca^{2+} , cAMP, NO, Ca^{2+} -dependent protein kinases, protein phosphatases and proteases in the kidney cortex cells.

5. Regucalcin regulates nuclear function

Regucalcin in the cytoplasm of kidney cells is demonstrated to localize into the nucleus, and it has been found to regulate nuclear function; regucalcin was found to localize in the nucleus of HA-regucalcin/phCMV2-transfected NRK52E cells using immunocytochemical and western blot analysis (39). The nuclear localization of regucalcin was enhanced after culture with FBS, PTH, Bay K 8644 or PMA (47). This enhancement was remarkable following culture with PMA, which is an activator of protein kinase C, and it was suppressed by staurosporine, an inhibitor of protein kinase C (47). Thus, the nuclear localization of regucalcin was enhanced through Ca^{2+} -signaling factors including protein kinase C in NRK52E cells (47). The stimulatory effect of PTH on regucalcin mRNA expression in NRK52E cells was mediated through cAMP or IP_3 -released Ca^{2+} and protein kinase C (12). Thus, it has been demonstrated that protein kinase C enhances both regucalcin mRNA expression and nuclear localization of regucalcin protein in NRK52E cells.

Regucalcin was found to inhibit the activity of various protein phosphatases, including the Ca^{2+} /calmodulin-dependent enzyme in the nuclei of rat kidney cortex *in vitro* (39,40).

Regucalcin has been shown to exert suppressive effects on DNA synthesis activity in the nuclei isolated from rat renal cortex *in vitro* (48). Such an effect was induced by adding of regucalcin (0.1-0.5 μM) to the reaction mixture (48). Also, the suppressive effects of regucalcin were noted in the presence of calcium chloride (50 μM) in the reaction mixture, and this suppression was potentiated in the presence of

EGTA (1 mM) (48). The suppressive effects of regucalcin on nuclear DNA synthesis were thus mediated through a mechanism unrelated to Ca^{2+} . The presence of anti-regucalcin monoclonal antibody (10-50 ng/ml) in the reaction mixture caused an increase in nuclear DNA synthesis activity (48). This increase was completely abolished by the addition of exogenous regucalcin (0.5 μM) (48). Regucalcin can directly bind to nuclear DNA and inhibit DNA synthesis (49). Nuclear regucalcin may play a role in the suppression of nuclear DNA synthesis in kidney proximal tubular epithelial cells (50).

Moreover, regucalcin, which was localized in the nucleus, has been found to regulate the gene expression of various proteins involved in ion transport (31), proliferation and apoptosis in kidney cells.

6. Suppressive effect of regucalcin on the proliferation of kidney cells

The overexpression of endogenous regucalcin was found to suppress the proliferation of NRK52E cells *in vitro* (30). The suppressive effects of regucalcin on cell proliferation were prevented following culture with butyrate, roscovitine and sulforaphane, which induce cell cycle arrest (30). Butyrate induces the inhibition of G1 progression (50). Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2 and cdk5 (51) and can lead to cell cycle arrest in G1 and accumulation in the G2 phase of the cell cycle. Sulforaphane can induce G2/M phase cell cycle arrest (52). The effect of butyrate, roscovitine or sulforaphane, which inhibit the proliferation of wild-type NRK52E cells, was not noted in the transfectants overexpressing regucalcin (29). These findings support the view that endogenous regucalcin induces G1 and G2/M phase cell cycle arrest in NRK52E cells.

The proliferation of NRK52E cells was also suppressed following culture with PD98059, staurosporine or dibucaine, which are inhibitors of various protein kinases (29). Such suppression was not observed in the transfectants that overexpressed endogenous regucalcin (29). The suppressive effects of endogenous regucalcin on cell proliferation were suggested to result from inhibitory effects of regucalcin on various protein kinases that are involved in the stimulation of cell proliferation. The suppressive effects of endogenous regucalcin on cell proliferation were shown to be mediated through the inhibition of PI3-kinase, using its inhibitor wortmannin (53). Moreover, the overexpression of regucalcin was shown to prevent the suppression of cell proliferation induced by Bay K 8644, an agonist of calcium entry into cells (29), supporting the view that endogenous regucalcin maintains intracellular Ca^{2+} homeostasis.

The overexpression of regucalcin was found to regulate the gene expression of proteins that are involved in cell proliferation and cell cycle. The expression of c-jun and checkpoint kinase 2 (chk2) mRNAs was suppressed by overexpression of regucalcin (29). The expression of p53 mRNA was enhanced by overexpression of regucalcin, while the expression of c-myc, c-fos, cdc2 and p21 mRNAs was not changed (29). The suppression of c-jun and chk2 mRNA expression may lead to retardation of cell proliferation. Regucalcin may exert suppressive effects on cell proliferation by regulating the gene expression of many proteins related to cell proliferation in NRK52E cells.

Thus, the suppressive effects of regucalcin on cell proliferation may be mediated through a decrease in various Ca^{2+} signaling-dependent protein kinases and PI3-kinase activities, the suppression of c-jun and chk2 mRNA expression, or the enhancement of p53 mRNA expression in kidney NRK52E cells.

7. Suppressive effect of regucalcin on apoptotic cell death

The role of endogenous regucalcin in apoptotic cell death has been demonstrated in NRK52E cells overexpressing regucalcin (22,54). The overexpression of regucalcin was found to prevent the apoptotic cell death of NRK52E cells induced by culture with TNF- α , TGF- β 1, lipopolysaccharide (LPS), Bay K 8644, or thapsigargin, suggesting the enhancement of nuclear DNA fragmentation (54). It has been suggested that the suppressive effect of regucalcin on apoptotic cell death is mediated through the suppression of various intracellular signaling pathways, which was induced by caspase-3, NO and Ca^{2+} in NRK52E cells (54).

Bcl-2 is a suppressor of apoptotic cell death (55). Apaf-1 participates in the activation of caspase-3 (56). Akt-1 is involved in survival signaling for cell death (56). The overexpression of regucalcin was found to cause a marked elevation of Bcl-2 mRNA expression in NRK52E cells, and it slightly stimulated Akt-1 mRNA expression in the cells (54). The overexpression of endogenous regucalcin prevented LPS-suppressed Bcl-2 mRNA expression and LPS-stimulated Apaf-1 mRNA expression, and it suppressed LPS-induced cell death in NRK52E cells (54). Caspase-3 mRNA expression in NRK52E cells was enhanced following culture with TNF- α (54). This enhancement was prevented by the overexpression of regucalcin (54). Culture with Bay K 8644 or thapsigargin increased caspase-3 mRNA expression in wild-type NRK52E cells, and these increases were prevented by regucalcin overexpression (54). Thus, overexpression of endogenous regucalcin enhances the expression of Bcl-2 and Akt-1 mRNAs, and it suppresses the expression of caspase-3 and Apaf-1 mRNAs in NRK52E cells, thereby inducing suppression of apoptotic cell death. Many toxic factors have been reported to induce renal failure by stimulating apoptotic cell death (57). Thus, endogenous regucalcin may play an important role as a suppressor protein in the promotion of apoptotic cell death and renal failure in kidney proximal tubule epithelial cells.

Smads are involved in the signal transduction of TGF- β 1 (58). NF- κ B is involved in the intracellular signaling of TNF- α (59). The expression of Smad 2 and NF- κ B mRNAs in wild-type NRK52E cells was enhanced by the overexpression of regucalcin (22), suggesting that regucalcin stimulates the gene expression of Smad 2 or NF- κ B. Of note, the overexpression of regucalcin was found to suppress α -smooth muscle actin expression in NRK52E cells (22). Culture with TNF- α or TGF- β 1 caused a marked increase in α -smooth muscle actin levels in NRK52E cells (22). This increase was suppressed by the overexpression of regucalcin (22). These findings suggest that endogenous regucalcin suppresses α -smooth muscle actin production in NRK52E cells cultured with TNF- α or TGF- β 1. TGF- β 1 is a key mediator that regulates transdifferentiation of NRK52E cells into myofibroblasts, which express α -smooth muscle actin (60). This is known to lead to renal fibrosis associ-

ated with overexpression of TGF- β 1 that is seen in the diseased kidney (60).

As summarized in Fig. 2, suppressed regucalcin gene expression may lead to apoptotic cell death and renal fibrosis, suggesting that regucalcin plays a pathophysiological role in kidney proximal tubule epithelial cells. Regucalcin exerts suppressive effects on proliferation and apoptotic cell death induced through various stimuli in kidney proximal tubule epithelial cells. Thus, it can be suggested that regucalcin may play a physiological role in maintaining cell homeostasis in kidney proximal tubular epithelial cells.

8. Involvement of regucalcin in kidney failure

Regucalcin gene expression has been shown to suppress kidney failure induced in various pathophysiologic states. Kidney regucalcin gene expression has been found to be suppressed in hypertensive states. Regucalcin expression has been shown to be suppressed in spontaneously hypertensive rats, suggesting the involvement of regucalcin in hypertensive states (61,62). Regucalcin mRNA expression was also suppressed in the kidney cortex of rats following saline administration for 7 days (61-63). This intake caused an increase in the serum calcium and blood urea nitrogen (BUN) concentrations, which are an index of kidney disorder (61,62), and it also caused an increase in Ca^{2+} -ATPase activity in the basolateral membranes of the kidney cortex and a corresponding increase in renal cortex calcium content (62). It is therefore suggested that suppressed regucalcin expression may lead to a disturbance of kidney calcium metabolism, which is related to renal hypertension.

Several drugs are known to cause nephrotoxicity. Cisplatin, a nephrotoxic antitumor drug (64), or cephaloridine, a nephrotoxic cephalosporin antibiotic (65), is known to change the thiol status in the renal cortex, and this takes place before significant morphological changes occur. Regucalcin mRNA expression and its protein levels were markedly reduced in the kidney cortex of rats which received a single intraperitoneal administration of cisplatin or cephaloridine, and this also induced a marked accumulation of calcium in the kidney cortex and a corresponding elevation of BUN (66,67). Moreover, regucalcin was downregulated by the administration of hexachloro-1:3-butadiene (HCBD) in low doses (68). Glutamine synthase activity in the kidney cortex was also downregulated, whereas BUN and creatinine levels increased after a high dose of HCBD (68). Regucalcin gene expression appears to be a sensitive genomic marker which can be used to evaluate the renal impairment caused by chemicals, and its downregulation seems to be related to damage of the proximal tubule (68).

Ochratoxin A (OTA), a naturally occurring mycotoxin, is nephrotoxic in all animal species investigated thus far and is considered a potent renal carcinogen, although the mechanism of its toxicity remains unknown (69). The gene expression profiles in target and non-target organs were analyzed after oral administration of OTA in rats (69). The number of differentially expressed genes in the kidney was much higher than those in the liver (541 vs. 11 at both time-points) (69). Downregulation was predominant in the genes involved in the oxidative stress response pathway, and metabolism and transport (69).

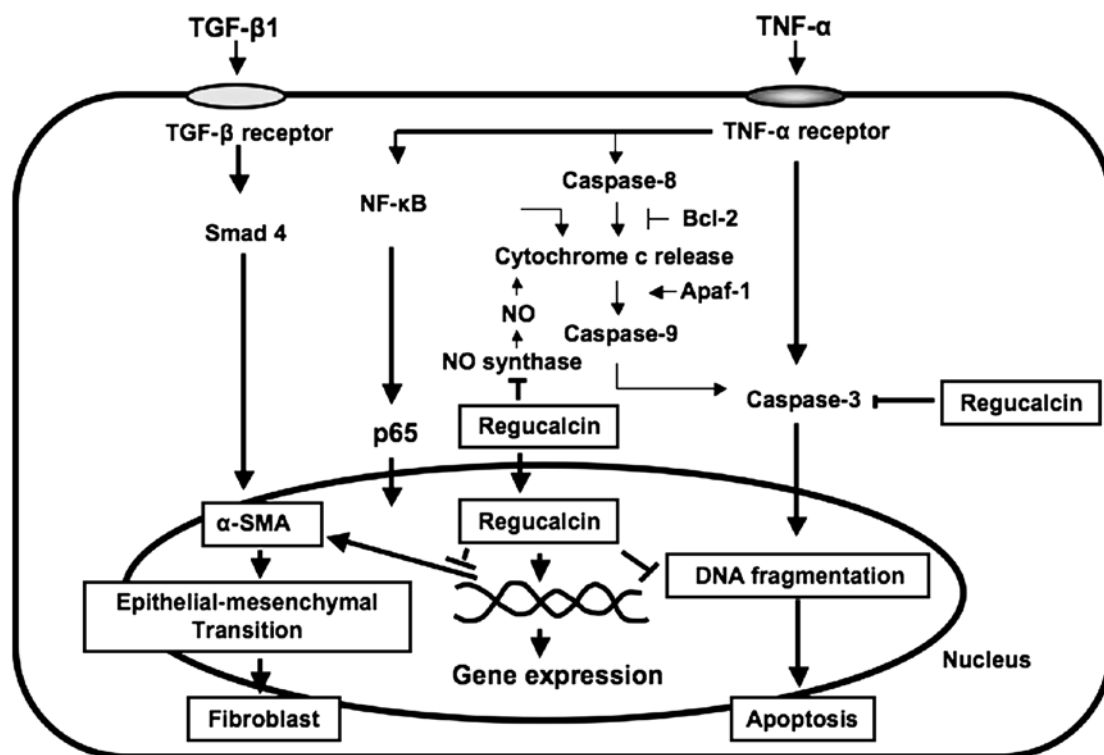


Figure 2. Endogenous regucalcin plays a pivotal role as a suppressor protein in the development of renal fibrosis and apoptotic cell death in the kidney proximal tubular epithelial cells. Transforming growth factor- β (TGF- β)1 or tumor necrosis factor- α (TNF- α) caused a remarkable increase in the expression of α -smooth muscle actin (α -SMA) in the kidney cells. TGF- β 1 is a key mediator that leads to myofibroblasts due to increasing α -SMA, leading to renal fibrosis associated with overexpression of TGF- β 1 within the diseased kidney. The expression of α -SMA was suppressed by overexpression of regucalcin. Moreover, overexpression of regucalcin suppresses apoptotic cell death induced by TNF- α due to inhibiting caspase-3 activity in the kidney cells. The suppressed regucalcin gene expression may lead to the development of apoptotic cell death and renal fibrosis. Bcl-2, B-cell lymphoma 2; apaf-1, apoptotic protease activating factor; NO, nitric oxide; NF- κ B; nuclear factor- κ B.

Regucalcin was strongly suppressed at both time points, while the genes implicated in cell survival and proliferation were upregulated at day 21, and translation factors and Annexin were upregulated at both time points (69).

The prolonged intake of aristolochic acid (AA) has been shown to be associated with the development of certain renal disorders in rats (70). Renal tubular atrophy and interstitial fibrosis are the early symptoms of AA nephropathy. Differentiated proteins have been identified in the kidney tissues through proteomics investigations (70). Upregulated proteins identified included ornithine aminotransferase, sorbitol dehydrogenase, actin, aspartoacylase, 3-hydroxyisobutyrate dehydrogenase and peroxiredoxin-1 (70). Downregulated proteins included regucalcin, ATP synthase subunit β , glutamate dehydrogenase 1, glutamate-cysteine ligase regulatory subunit, dihydropteridine reductase, hydroxyacyl-coenzyme A dehydrogenase, voltage-dependent anion-selective channel protein 1, prohibitin and adenylate kinase isoenzyme 4 (70). Thus, these identified protein markers are suggested to have biological and medical significance.

The basic mechanism underlying calcineurin inhibitor (cyclosporine) nephrotoxicity with enhancement by sirolimus is still largely unknown (71). The effects of cyclosporine, alone and in combination with sirolimus, on the renal proteome have been investigated (71). Twenty-four-hour urine was collected for nuclear magnetic resonance (NMR)-based metabolic analysis, and the kidneys were harvested for two dimensional-gel electrophoresis and histology (71).

Cyclosporine affected the following groups of proteins: calcium homeostasis (regucalcin and calbindin), cytoskeleton (vimentin and caldesmon), response to hypoxia and mitochondrial function [prolyl 4-hydroxylase, proteasome and reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase], and cell metabolism (kidney aminoacylase, pyruvate dehydrogenase and fructose-1, 6-bisphosphate) (71). It was noted that regucalcin was found to be increased in the urine of rats with kidney disorder, indicating that regucalcin is a useful potential biomarker of kidney disorders (71).

As described above, the suppression of regucalcin gene expression and its association with nephrotoxicity may play a pathophysiological role in the development of renal disorders.

9. Prospects

Ca^{2+} , which plays a multifunctional role in many biological systems, plays a pivotal role during embryonic development of kidneys, and it is present throughout numerous steps of tubulogenesis and nephron induction, from the formation of a simple kidney in amphibian larvae (pronephros) to the formation of the more complex mammalian kidney (metanephros) (2). Regucalcin, a regulatory protein of Ca^{2+} signaling in kidney cells, plays a pivotal role in the development of the kidneys, as it is involved in tubulogenesis and also in nephron induction.

Regucalcin has been demonstrated to play a multifunctional role in the regulation of intracellular Ca^{2+} transport, the activity of various cell signaling-related enzymes, nuclear DNA

synthesis, gene expression, proliferation and apoptotic cell death in kidney cells. Regucalcin gene expression was found to be suppressed in various pathophysiological conditions including hypertensive states, and nephrotoxics are associated with kidney failure. An analysis for proteome and differential gene expression demonstrated a potential suppression of regucalcin expression among many proteins. Thus, it can be suggested that suppressed regucalcin gene expression may lead to development of renal failure. The pathophysiological role of regucalcin in kidney diseases in human subjects is poorly understood. Of note, regucalcin gene expression and its protein have been shown to be suppressed in kidney tumor tissues as compared with kidney tissues of healthy humans (72), suggesting that regucalcin plays a suppressive role in the development of carcinogenesis in the kidneys of human subjects. Clinical studies on regucalcin are thus necessary in order to examine its role further.

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