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# Suppressive Role of Endogenous Regucalcin in the Enhancement of Nitric Oxide Synthase Activity in Liver Cytosol of Normal and Regucalcin Transgenic Rats

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**Abstract** The suppressive role of endogenous regucalcin, which is a regulatory protein of calcium signaling, in the enhancement of nitric oxide (NO) synthase activity in the liver cytosol of rats was investigated. The enzyme activity was measured in a reaction mixture containing either vehicle or calcium chloride (1–20 μM) in the absence or presence of regucalcin (0.1, 0.25, or 0.5 μM). NO synthase activity was significantly increased by the addition of calcium (5–20 μM). This increase was completely abolished in the presence of trifluoperazine (TFP; 10–50 μM), an antagonist of $Ca^{2+}$/calmodulin. The addition of regucalcin (0.1–0.5 μM) caused a significant fall in the calcium-increased enzyme activity. The effect of regucalcin (0.25 μM) in decreasing NO synthase activity was seen in the presence of ethylene glycol bis-(2-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 1 mM) or TFP (20 μM), indicating that regucalcin acts independent on $Ca^{2+}$/calmodulin. NO synthase activity was significantly raised in the presence of anti-regucalcin monoclonal antibody (10–50 ng/ml) in the reaction mixture. The effect of the antibody (50 ng/ml) or calcium (10 μM) in elevating NO synthase activity in the liver cytosol of normal rats was not seen in the liver cytosol obtained from regucalcin transgenic rats. Moreover, the increase in NO synthase activity in the liver cytosol of normal rats induced by a single intraperitoneal administration of calcium (5.0 mg/100 g body weight) was significantly enhanced in the presence of anti-regucalcin monoclonal antibody (50 ng/ml) in the reaction mixture. The administration of calcium caused a significant increase in regucalcin level in the liver cytosol of normal rats. The present study demonstrated that endogenous regucalcin plays a suppressive role in the enhancement of NO synthase activity in the liver cytosol of rats. J. Cell. Biochem. 88: 1226–1234, 2003. 

**Key words:** regucalcin; nitric oxide synthase; calcium; liver; transgenic rats

Calcium ion ($Ca^{2+}$) plays a pivotal role in the regulation of many cell functions. The $Ca^{2+}$ effect in cells is amplified by $Ca^{2+}$-dependent protein kinases, which are related to a signal transduction due to hormonal stimulation [Cheung, 1980; Williamson et al., 1981; Reinhart et al., 1984; Kraus-Friedman and Feng, 1996]. Regucalcin, which was found as a novel $Ca^{2+}$-binding protein not including the EF-hand motif [Yamaguchi and Yamamoto, 1978; Shimokawa and Yamaguchi, 1993], has been demonstrated to play a multifunctional role as a regulatory protein in $Ca^{2+}$-signaling process in cells [Yamaguchi, 2000a,b; reviews].

Regucalcin is predominantly present in the liver of rats [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The role of regucalcin in liver cells is demonstrated. It has been shown that regucalcin can regulate intracellular $Ca^{2+}$ homeostasis [Takahashi and Yamaguchi, 1994, 1999, 2000] in rat liver, and that it has an inhibitory effect on $Ca^{2+}$-dependent protein kinases [Katsumata and Yamaguchi, 1998; Yamaguchi and Katsumata, 1999] and protein phosphatase [Omura and Yamaguchi, 1998, 1999] in rat liver cells. Moreover, regucalcin has been shown to suppress overexpression of cell proliferation due to inhibiting nuclear deoxyribonucleic acid and ribonucleic acid syntheses in rat liver cells

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[Yamaguchi and Ueoka, 1997; Misawa et al., 2002; Tsurusaki and Yamaguchi, 2002]. Regucalcin in the cytoplasm is translocated to the nucleus and regulates nuclear functions in rat liver cells [Tsurusaki et al., 2000; Yamaguchi, 2000b]. Regucalcin may play a pivotal role as a regulatory protein in the regulation of cell function in liver cells.

Nitric oxide (NO) may be important as a signaling factor in many cells [Lowenstein et al., 1994; Svhmidt and Walter, 1994]. NO, which an unpaired electron reacts with protein, targets primarily through their thiol or heme groups, and 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 catalyzed by NO synthase that requires NADPH, $Ca^{2+}$/calmodulin, and others [Lowenstein et al., 1994]. The effect of regucalcin on NO synthase activity in the liver cytosol of rats has not been clarified so far.

The present study, therefore, was undertaken to determine the effect of regucalcin on NO synthase activity in rat liver cytosol. We found that endogenous regucalcin has a suppressive effect on NO synthase activity in the liver cytosol of normal rats, regucalcin transgenic (TG) rats, or calcium-administered rats.

## MATERIALS AND METHODS

### Chemicals

Arginine, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), citrulline, calmodulin (52,000 units/mg protein from bovine brain), trifluoperazine (TFP), and ethylene glycol bis-(2-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, pH 7.0) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride and other chemicals were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Reagents used were dissolved in distilled water, and some reagents were passed through ion-exchange resin to remove metal ions.

### Animals

Male and female Wistar rats (4-week old) were used. They were obtained commercially from Japan SLC, Inc. (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus at a room temperature of 25°C and were allowed distilled water freely.

### Regucalcin TG Rats

Regucalcin TG rats were generated previously [Yamaguchi et al., 2002]. The TG rats were obtained from Japan SLC Inc. To determine transgene copy number, genomic DNA was isolated from tails of TG rats and amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primer sets that recognized two different regions of the regucalcin cDNA [Misawa and Yamaguchi, 2000]. Primers huRC-1 (5′-GGAGGCTATGTT GCCACCATT-GGA-3′) and huRC-2 (5′-CCCTCCAAAGC-AGCATGAAGTTG-3′) amplified a fragment containing the regucalcin cDNA that was present in the transgene sequence, but absent in the wild-type (wt). Regucalcin TG rats were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus at a room temperature of 25°C and were allowed distilled water freely.

### Isolation of Regucalcin

Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993] from which it was isolated. The livers were perfused with Tris-HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4°C. The livers were removed, cut into small pieces, suspended 1:4 (weight:volume) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000*g* for 60 min. The resulting supernatant was heated at 60°C for 10 min and re-centrifuged at 38,000*g* for 20 min. Regucalcin in the supernatant was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously [Yamaguchi and Yamamoto, 1978].

### Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods [Omura and Yamaguchi, 1998]. Mouse (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg/animal of antigen (rat liver RC) emulsified with Freund's complete adjuvant, and 19 days later antigen (0.25 mg/animal) was intraperitoneally (i.p.) injected with Freund's incomplete adjuvant. Animals were sacrificed by bleeding

3 days after the last injection. Spleen cells were prepared from immuned mouse, and fused into myeloma cells. Anti-regucalcin monoclonal antibody-producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through a protein A-agarose column (Sigma).

### Calcium Administration

Calcium chloride was dissolved in sterile distilled water as a concentration of 5 mg Ca/ml. The solution (0.5 ml/100 g body weight of rats) was i.p. administered to normal rats, and the animals were killed by bleeding at 60 min after calcium administration.

### Preparation of Subcellular Fraction

Liver was perfused with ice-cold 0.25 M sucrose solution and immediately removed, cut into small pieces, suspended (1:4) in 0.25 M sucrose solution and homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 600*g* in a refrigerated centrifuge for 10 min and the supernatant was spun at 5500*g* for 10 min to obtain the mitochondria. The 5500*g* supernatant was spun at 1,05,000*g* for 60 min, and the supernatant fraction (cytosol) and the resulting pellets (microsomes) were pooled.

The plasma membranes from liver were prepared according to the procedure of Prpic et al. [1984]. Livers were minced with scissors and homogenized by 10 passes with a loose-fitting Dounce homogenizer followed by three passes with a tight-fitting homogenizer, then diluted to give a 6% (w/v) homogenate. The homogenate was then centrifuged at 1,464*g* for 10 min, and the resulting pellet was resuspended in the isolation medium and diluted to give a 6% (w/v) suspension. A volume (10.4 ml) of this was mixed with 1.4 ml of Percoll (Pharmacia) in 15 ml Cortex tubes and centrifuged at 34,540*g* for 30 min. Two distinct layers close to the top of the tube were revealed. These were harvested and washed in 5 volumes of 0.25 M sucrose, 50 mM Tris-HCl, pH 8.0 and the resulting pellets (plasma membranes) were resuspended in the same medium. Assay of marker enzyme (5′-nucleotidase, succinate dehydrogenase, glucose-6-phosphatase, and RNA polymerase) showed that there was very little contamination by nuclei, mitochondria, or microsomes. Especially, the activity of plasma membrane 5′-nucleotidase showed a high value in comparison with the activity of other enzymes.

The nuclei from liver were isolated by the procedure of Jones et al. [1989] with a minor modification. Liver was homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in 40 ml of TKM solution (50 mM Tris-HCl, pH 4.5, 25 mM KCl, 5 mM $MgCl_2$) containing 0.25 M sucrose and 1.0 mM EGTA. The homogenate was filtered through three layers of cheesecloth. Nuclei were pelletized by centrifugation at 700*g* for 10 min. The pellets were homogenized (five strokes) in 40 ml of TKM solution and centrifuged again at 700*g* for 10 min. The pellets were resuspended in 24 ml of TKM solution by homogenization (five strokes), and 6 ml of the resuspended pellets was added to each of four tubes containing 12 ml of TKM including 2.3 M sucrose. The tubes were gently mixed, and a 6-ml cushion (TKM containing 2.3 M sucrose) was carefully layered on the bottom of each tube. The tubes were centrifuged at 37,000*g* for 30 min. The upper layer and the sucrose cushion were removed with an aspirator. The resulting pellet of highly purified nuclei was resuspended in ice-cold 0.25 M sucrose solution by hand homogenization. Assay of marker enzymes showed that there was less than 5% contamination by microsomes, plasma membranes, or mitochondria. DNA content in the nuclei was determined using the diphenylamine reaction [Burton, 1956].

Protein concentration in the subcellular fraction was determined by the method of Lowry et al. [1951] using bovine serum albumin as the standard.

### Assay of NO Synthase

NO synthase activity in the cytosol of liver 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 cytosolic protein (650–850 μg/ml) in the absence or presence of calmodulin (2.5 μg/ml). In separate experiments, the reaction mixture contained either vehicle, calcium chloride (1.0–20 μM), regucalcin (0.1, 0.25, or 0.5 μM), anti-regucalcin monoclonal antibody (10–50 ng/ml) or other reagents. The enzyme reaction was terminated by the addition of 1.0 ml of cold 10% trichloroacetic acid and

centrifuged to precipate protein. Produced citrullin in the supernatant was quantified by the method of Boyde and Rahmatullah [1980]. Results were expressed as nanomoles of citrullin produced per minute per milligram (mg) of cytosolic protein.

### Western Blot Analysis

The subcellular fractions from liver homogenate were used for Western blot analysis [Wessendorf et al., 1993; Tsurusaki et al., 2000]. Aliquots of subcellular fraction containing 43 or 86 μg of protein, were mixed with 5× sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 12% polyacrylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-RC antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% (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-rabbit IgG, which was diluted 1:5,000 with washing buffer containing 5% (w/v) skim milk, and washed again. Detection of the protein bands was performed using an enhanced chemiluminescent kit (Amersham, Buckinghamshire, UK) following the manufacture's instructions. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel. The density of protein bands was quantified by densitometer scanning (Dual wavelength Flying-spot Scanner CS-9000, Shimadzu Company, Japan).

### Statistical Analysis

Data were expressed as the mean ± SEM. The significance of the difference between the values was estimated by Student's *t*-test. We also used a multiway ANOVA comparison test to compare the treatment groups. A *P*-value of $<0.05$ was considered to indicate a statistically significant difference.

## RESULTS

### Effect of Regucalcin on NO Synthase Activity in the Liver Cytosol of Normal Rats

The effect of calcium chloride addition on NO synthase activity in the liver cytosol of normal rats is shown in Figure 1. The enzyme activity was measured in a reaction mixture containing either vehicle or calcium chloride (1, 5, 10, or 20 μM) (Fig. 1A). NO synthase activity was significantly increased in the presence of calcium chloride (5, 10, or 20 μM). When calmodulin (2.5 or 5.0 μg/ml) was added into the enzyme reaction mixture containing calcium chloride (10 μM), NO synthase activity was not significantly enhanced as compared with that of calcium (10 μM) addition (Fig. 1B).

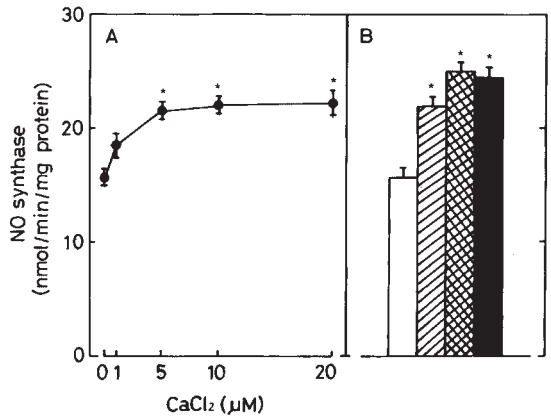


**Fig. 1.** Effect of calcium chloride addition on NO synthase activity in the liver cytosol of normal rats. **A**: The enzyme activity was measured in a reaction mixture containing either vehicle or calcium chloride (1, 5, 10, or 20 μM). **B**: The reaction mixture contained either vehicle or calmodulin (2.5 or 50 μg/ml) in the absence or presence of calcium chloride (10 μM). Each value is the mean ± SEM of five rats. $^*P < 0.01$, compared with the control (none) value. White bar, control (none); hatched bar, calcium chloride; double hatched bar, calcium chloride plus calmodulin (2.5 μg/ml); black bar, calcium chloride plus calmodulin (5.0 μg/ml).

The effect of TFP, an antagonist calmodulin [Vincenzi, 1982], on the calcium addition-increased NO synthase activity in the liver cytosol of normal rats is shown in Figure 2. The enzyme reaction mixture contained either vehicle or calcium chloride (10 μM) in the absence or presence of TFP (10, 20, or 50 μM). NO synthase activity was significantly increased by the addition of calcium chloride (10 μM) (Fig. 2A). This increase was completely abolished in the presence of TFP (10, 20, or 50 μM), or antagonist of calmodulin (Fig. 2B). This result indicates that the effect of calcium addition in increasing NO synthase activity is related to endogenous calmodulin in liver cytosol.

The effect of regucalcin addition on NO synthase activity in the liver cytosol of normal rat

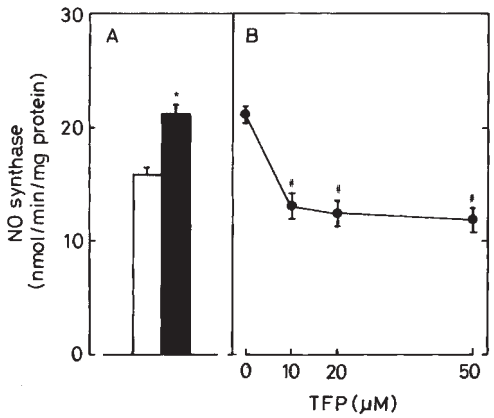


**Fig. 2.** Effect of TFP on the calcium chloride-increased NO synthase activity in the liver cytosol of normal rats. **A**: The enzyme activity was measured in a reaction mixture containing either vehicle or calcium chloride (10 μM). **B**: The reaction mixture contained either vehicle or TFP (10, 20, or 50 μg/ml) in the presence of calcium chloride (10 μM). Each value is the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) value. #$P < 0.01$, compared with the value obtained by calcium addition. White bar, control (none); black bar, calcium addition.

liver is shown in Figure 3. The enzyme reaction mixture contained either vehicle or regucalcin (0.1, 0.25, or 0.5 μM) in the absence or presence of calcium chloride (10 μM). The addition of regucalcin (0.1, 0.25, or 0.5 μM) caused a significant decrease in the calcium addition-increased NO synthase activity (Fig. 3A) in liver cytosol (Fig. 3B).

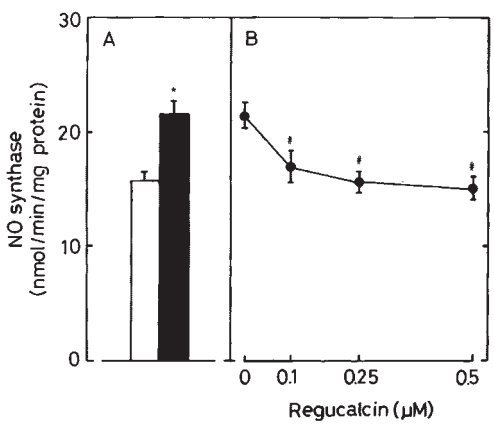


**Fig. 3.** Effect of regucalcin addition on NO synthase activity in the liver cytosol of normal rats. **A**: The enzyme activity was measured in a reaction mixture containing either vehicle or calcium chloride (10 μM). **B**: The reaction mixture contained either vehicle or regucalcin (0.1, 0.25, or 0.5 μM) in the presence of calcium chloride (10 μM). Each value is the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) value. #$P < 0.01$, compared with the value obtained by calcium addition. White bar, control (none); black bar, calcium addition.

Regucalcin (0.1 or 0.25 μM) was added to the enzyme reaction mixture containing EGTA (1 mM) or TFP (20 μM) without calcium addition (Fig. 4). NO synthase activity in liver cytosol was not significantly altered by the presence of EGTA or TFP. The addition of regucalcin (0.25 μM) caused a significant decrease in NO synthase activity in the presence of EGTA or TFP. This result indicates that the effect of regucalcin in decreasing NO synthase activity in liver cytosol is independent on $Ca^{2+}$/calmodulin.

The effect of anti-regucalcin monoclonal antibody on NO synthase activity in the liver cytosol of normal rats is shown in Figure 5. The enzyme activity was measured in a reaction mixture containing either vehicle or anti-regucalcin monoclonal antibody (10, 25, or 50 ng/ml) in the presence of EGTA (1 mM) or calcium chloride (10 μM). NO synthase activity was significantly increased by the addition of anti-regucalcin monoclonal antibody (10, 25, or 50 ng/ml) in the presence of EGTA or calcium chloride. From this result, it was suggested that endogenous regucalcin in liver cytosol has a suppressive effect on NO synthase activity.

### Role of Endogenous Regucalcin on NO Synthase Activity in the Liver Cytosol of Regucalcin TG Rats

The change in endogenous regucalcin in the liver cytosol of regucalcin TG rats is shown in Figure 6. The result of Western analysis showed

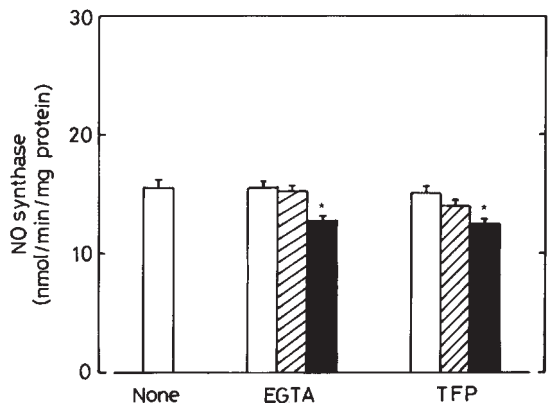


**Fig. 4.** Effect of regucalcin addition on NO synthase activity in the presence of EGTA or TFP in the liver cytosol of normal rats. The enzyme activity was measured in a reaction mixture containing either vehicle or regucalcin (0.1 or 0.25 μM) in the absence or presence of EGTA (1 mM) or TFP (20 μM). Each value is the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) value. White bar, control; hatched bar, regucalcin (0.1 μM); black bar, regucalcin (0.25 μM).

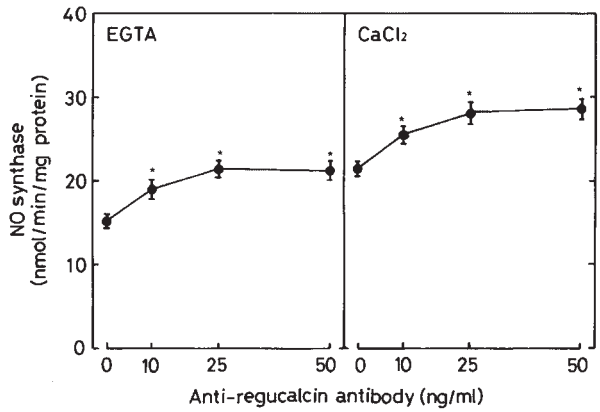


**Fig. 5.** Effect of anti-regucalcin monoclonal antibody on NO synthase activity in the liver cytosol of normal rats. The enzyme activity was measured in a reaction mixture containing either or anti-regucalcin monoclonal antibody (10, 25, or 50 ng/ml) in the presence of EGTA (1 mM) or calcium chloride (10 μM). Each value is the mean ± SEM of five rats. $*P < 0.01$, compared with the control (none) value.

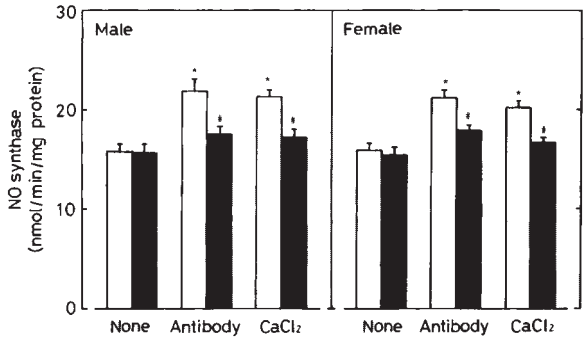


**Fig. 7.** Effect of anti-regucalcin monoclonal antibody or calcium chloride addition on NO synthase activity in the liver cytosol of wild-type and regucalcin TG rats. The enzyme activity was measured in a reaction mixture containing either vehicle, anti-regucalcin monoclonal antibody (50 ng/ml), or calcium chloride (10 μM). Each value is the mean ± SEM of five rats. $*P < 0.01$, compared with the control (none) value. $\#P < 0.01$, compared with the value obtained from wild-type rats. White bar, wild-type rats; black bar, TG rats.

that regucalcin was markedly expressed in the liver cytosol of male and female regucalcin TG rats as compared with that of wild-type rats.

The alteration in NO synthase activity in the liver cytosol of regucalcin TG rats is shown in Figure 7. NO synthase activity was measured in a reaction mixture containing either vehicle, anti-regucalcin monoclonal antibody (50 ng/ml), or calcium chloride (10 μM). NO synthase activity was not significantly altered in the liver cytosol of male and female regucalcin TG rats as compared with that of wild-type rats. The presence of anti-regucalcin monoclonal antibody (50 ng/ml) or calcium chloride (10 μM) in the enzyme reaction mixture caused a significant increase in NO synthase activity in the liver cytosol of wild-type rats. The effect of the antibody (50 ng/ml) or calcium chloride (10 μM) in increasing NO synthase activity was not seen in the liver cytosol of regucalcin TG rats.

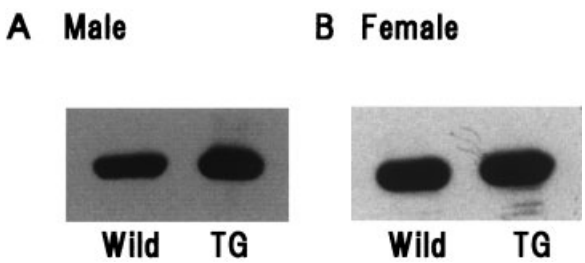


**Fig. 6.** Analysis of regucalcin protein in the liver cytosol of regucalcin TG rats. Western blot analysis was carried out on the cytosol (10 μg of cytosolic proteins). The figure shows one of four experiment with separate rats.

### Suppressive Effect of Endogenous Regucalcin on NO Synthase Activity in the Liver Cytosol of Calcium-Administrated Normal Rats

The localization of regucalcin protein in the plasma membranes, mitochondria, microsomes, nucleus, and cytoplasm of liver in normal rats is shown in Figure 8. Regucalcin was present in the plasma membranes, mitochondria, microsomes, nucleus, and cytoplasm of normal rat liver. Especially, regucalcin was greatly found in the cytoplasm of rat liver. Calcium chloride

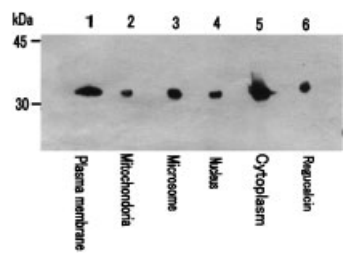


**Fig. 8.** Analysis of regucalcin protein in the subcellular fraction of normal rat liver. Western blot analysis was carried out on the extracts (86 μg of each subcellular fraction) obtained from the liver homogenate of normal rats. Regucalcin (0.1 μg) as the marker was used in **lane 6**. The membranes were exposed on film for 30 s. The figure shows one of the four experiments with separate rats. The densitometric data showed: 59 ± 4.7 (plasma membranes), 17 ± 3.1 (mitochondria), 35 ± 2.9 (microsomes), or 22 ± 2.0 (nucleus) (percent of the value obtained from cytoplasm; mean ± SEM of four rats).

(5.0 mg Ca/100 g body weight) was i.p. administered to rats, and 60 min later the animals were killed by bleeding. Regucalcin levels in the plasma membranes, mitochondria, microsomes, nucleus, and cytoplasm of the liver were significantly ($P < 0.01$) increased by the administration of calcium chloride to rats (Fig. 9).

The change in NO synthase activity in the liver cytosol of calcium-administered rats is shown in Figure 10. Rats were killed by bleeding at 60 min after a single i.p. administration of calcium chloride (5.0 mg Ca/100 g body weight) to normal rats. The enzyme activity was measured in a reaction mixture containing either vehicle or anti-regucalcin monoclonal antibody (100 ng/ml). NO synthase activity was significantly raised by the administration of calcium. This increase was significantly enhanced in the presence of anti-regucalcin monoclonal antibody, indicating a suppressive role of endogenous regucalcin in the regulation of NO synthase activity.

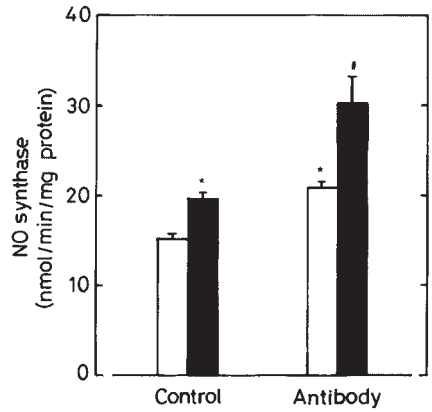


**Fig. 10.** Effect of anti-regucalcin monoclonal antibody on NO synthase activity in the liver cytosol of normal or calcium-administered rats. Rats received a single i.p. administration of calcium chloride (5 mg Ca/100 g body weight), and they were killed 60 min after the administration. The enzyme activity was measured in a reaction mixture containing either vehicle or anti-regucalcin monoclonal antibody (100 ng/ml). Each value is the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none). #$P < 0.01$, compared with the value (control) obtained from calcium-administered rats. White bars, normal rats; black bars, calcium-administered rats.

## DISCUSSION

The present study demonstrates that regucalcin has an inhibitory effect on NO synthase activity in the liver cytosol of rats. NO synthase activity in the liver cytosol was significantly increased by the addition of calcium, and this increase was completely prevented in the presence of TFP, an antagonist of calmodulin [Vincenzi, 1982]. The effect of calcium addition in increasing NO synthase activity is mediated through calmodulin, indicating the existence of $Ca^{2+}$/calmodulin-dependent NO synthase activity in rat liver cytosol.

Regucalcin was found to inhibit NO synthase activity in the liver cytosol of rats. This is a novel finding. The effect of regucalcin in decreasing liver cytosolic NO synthase activity was seen in the presence of EGTA, a chelator of $Ca^{2+}$, or TFP. This result suggests that regucalcin can directly inhibit NO synthase activity. In addition, regucalcin has been shown to bind to calmodulin [Omura and Yamaguchi, 1998]. Regucalcin may also have an inhibitory effect on the action of $Ca^{2+}$/calmodulin in activating NO synthase in rat liver cytosol. Regucalcin may have cumulative effect. The present finding, that regucalcin can decrease NO synthase activity, suggests that regucalcin plays a role

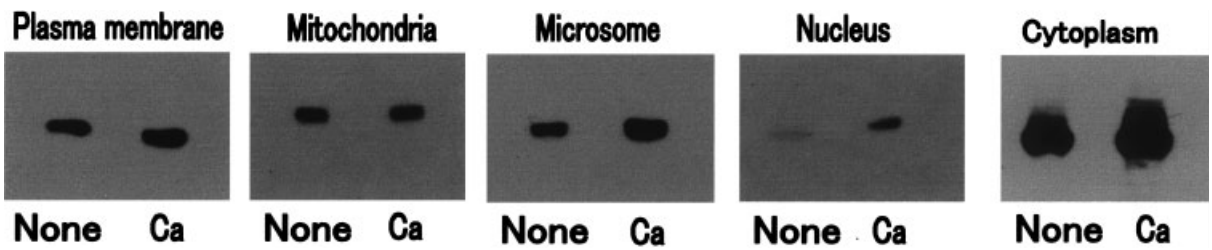


**Fig. 9.** Analysis of regucalcin protein in the subcellular fraction of the liver in normal or calcium-administered rats. Rats received a single i.p. administration of calcium chloride (5 mg Ca/100 g body weight), and they were killed 60 min after the administration. Western blot analysis was carried out on the extracts (43 μg of each subcellular fraction) obtained from the liver homogenate of normal or calcium-administered rats. The membranes were exposed on film for 1 min. The figure shows one of the four experiments with separate rats. The densitometric data showed a significant ($P < 0.01$) increase as compared with the control: 131 ± 4.9 (plasma membranes), 129 ± 3.5 (mitochondria), 181 ± 15.2 (microsomes), 266 ± 18.5 (nucleus), or 148 ± 6.8 (cytoplasma) (percent of control; mean ± SEM of four rats).

in the regulation of cellular function related to NO in liver cells.

The role of endogenous regucalcin in the regulation of NO synthase activity in rat liver cytosol was demonstrated by using anti-regucalcin monoclonal antibody. Liver cytosolic NO synthase activity was found to be increased in the presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture, suggesting an involvement of endogenous regucalcin. This was furthermore confirmed in regucalcin TG rats [Yamaguchi et al., 2002]. NO synthase activity was not significantly altered in the liver cytosol of regucalcin TG rats as compared with that of normal (wild-type) rats. However, the effect of anti-regucalcin monoclonal antibody or calcium addition in increasing NO synthase activity in normal rat liver cytosol was not seen in the liver cytosol of regucalcin TG rats, which overexpress regucalcin protein. These results suggest that endogenous regucalcin has a suppressive effect on NO synthase activity in rat liver cytosol.

The expression of hepatic regucalcin mRNA has been shown to be increased by a single i.p. administration of calcium chloride to normal rats [Shimokawa and Yamaguchi, 1992]. The present result showed that calcium administration induced a significant increase in regucalcin protein level in rat liver cytosol. NO synthase activity was significantly increased in the liver cytosol of calcium-administered rats. This elevation was significantly enhanced in the presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture. The result may further support the endogenous regucalcin has a suppressive effect on the enhancement of NO synthase activity in rat liver cytosol.

The existence of regucalcin in the subcellular fraction of normal rat liver was examined by using Western analysis. Regucalcin was showed to be greatly present in the cytosplasm of liver cells, and also it was present in the plasma membranes, mitochondria, microsomes, and nucleus. Radioiodinated regucalcin has been demonstrated to bind to those subcellular fractions [Yamaguchi et al., 1988]. This binding does not alter in the presence of $Ca^{2+}$. The administration of calcium has been shown to induce a significant increase in regucalcin content in rat liver [Isogai and Yamaguchi, 1995]. The present study founds that regucalcin levels in the subcellular fraction of rat liver was significantly increased by calcium administration. Regucalcin may play a suppressive role in the regulation of subcellular function related to $Ca^{2+}$ which is raised in liver cells due to hormonal stimulation and calcium intake.

Regucalcin has been demonstrated to play as a regulatory protein of NO synthase in the cytoplasm of liver cells. The suppressive effect on NO synthase activity in the liver cytosol of rats may be physiologic significance, since NO may act as a messenger or modulator molecule in liver cells [Lowenstein et al., 1994]. NO synthase is activated by $Ca^{2+}$/calmodulin which is related to $Ca^{2+}$ signaling. Regucalcin can inhibit $Ca^{2+}$ signaling in liver cells [Yamaguchi, 2000a; review]. Moreover, the overproduction of NO may lead to the damage of liver cells. Regucalcin may have a suppressive effect on overproduction of NO in liver cells, and the protein may have a protective effect on NO-induced damage of liver cells. Presumably, regucalcin plays a pathophysiologic role in health care of liver.

In conclusion, it has been demonstrated that endogenous regucalcin has a suppressive effect on the enhancement of NO synthase activity in the liver cytosol of rats. Regucalcin may play an important role as a regulatory protein of NO synthase in liver cells. This finding is novel.

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## REFERENCES

Boyde TRC, Rahmatullah M. 1980. Optimization of conditions for the colorimetric determination of citrulline, using diacetylmonoxine. Anal Biochem 107:424–431.

Burton A. 1956. Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of dioxyribonucleic acid. Biochem J 62:315–323.

Cheung WY. 1980. Calmodulin plays a pivotal role in cellular regulation. Science 202:19–27.

Isogai M, Yamaguchi M. 1995. Calcium administration increases calcium-binding protein regucalcin concentration in the liver of rats. Mol Cell Biochem 143:53–58.

Jones DP, McConkey DJ, Nicotera P, Orrenius S. 1989. Calcium activated DNA fragmentation in rat liver nuclei. J Biol Chem 264:6398–6403.

Katsumata T, Yamaguchi M. 1998. Inhibitory effect of calcium-binding protein regucalcin on protein kinase activity in the nuclei of regenerating rat liver. J Cell Biochem 71:569–576.

Kraus-Friedman N, Feng L. 1996. The role of intracellular $Ca^{2+}$ in the regulation of gluconegenesis. Metabolism 42:389–403.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 22:680–685.

Lee S-J, Stull JT. 1998. Calmodulin-dependent regulation of inducible and neuronal nitric-oxide synthase. J Biol Chem 273:27430–27437.

Lowenstein CJ, Dinerman JL, Snyder SH. 1994. Nitric oxide: A physiologic messenger. Ann Intern Med 120:227–237.

Lowry OH, Rosebrough NH, Farr AL, Randall RF. 1951. Protein measurement with the Folin plenol reagent. J Biol Chem 193:265–273.

Misawa H, Yamaguchi M. 2000. The gene of $Ca^{2+}$-binding protein regucalcin is highly conserved in vertebrate species. Int J Mol Med 6:191–196.

Misawa H, Inagaki S, Yamaguchi M. 2002. Suppression of cell proliferation and deoxyribonucleic acid synthesis in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84:143–149.

Omura M, Yamaguchi M. 1998. Inhibition of $Ca^{2+}$/calmodulin-dependent phosphatase activity by regucalcin in rat liver cytosol: Involvement of calmodulin binding. J Cell Biochem 71:140–148.

Omura M, Yamaguchi M. 1999. Enhancement of neutral phosphatase activity in the cytosol and nuclei of regenerating rat liver: Role of endogenous regucalcin. J Cell Biochem 73:332–341.

Prpic V, Green KC, Blackmore PF, Exton JH. 1984. Vassopressin-, angiotensin II-, and $\alpha_1$-adrenergic-induced inhibition of $Ca^{2+}$ transport by rat liver plasma membrane vesicles. J Biol Chem 259:1382–1385.

Reinhart PH, Taylor WM, Bygrave FL. 1984. The role of calcium ions in the mechanisms of action of $\alpha$-adrenergic agonists in rat liver. Biochem J 223:1–13.

Shimokawa N, Yamaguchi M. 1992. Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver. FEBS Lett 305:151–154.

Shimokawa N, Yamaguchi M. 1993. Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver. FEBS Lett 327:251–255.

Svhmidt HH, Walter U. 1994. NO at work. Cell 78:919–925.

Takahashi H, Yamaguchi M. 1994. Activating effect of regucalcin on ($Ca^{2+}$–$Mg^{2+}$)-ATPase in rat liver plasma membranes: Relation to sulfhydryl group. Mol Cell Biochem 136:71–76.

Takahashi H, Yamaguchi M. 1999. Role of regucalcin as an activator of $Ca^{2+}$-ATPase activity in rat liver microsomes. J Cell Biochem 74:663–669.

Takahashi H, Yamaguchi M. 2000. Stimulatory effect of regucalcin on ATP-dependent $Ca^{2+}$ uptake activity in rat liver mitochondria. J Cell Biochem 78:121–130.

Tsurusaki Y, Yamaguchi M. 2002. Suppressive role of endogenous regucalcin in the enhancement of deoxyribonucleic acid synthesis activity in the nucleus of regenerating rat liver. J Cell Biochem 85:516–522.

Tsurusaki Y, Misawa H, Yamaguchi M. 2000. Translocation of regucalcin to rat liver nucleus: Involvement of nuclear protein kinase and protein phosphatase regulation. Int J Mol Med 6:655–660.

Vincenzi FE. 1982. Pharmacology of calmodulin antagonism. In: Godfraind T, Alberfini A, Paoletti R, editors. Calcium modulators. Amsterdam: Elsevier Biomedical Press. pp 67–80.

Wessendorf JHM, Ganfinkel S, Zhan X, Brown S, Maciag T. 1993. Identification of a nuclear localization sequence within the structure of the human interleukin-1 $\alpha$ precursor. J Biol Chem 268:22100–22104.

Williamson JR, Cooper RK, Hoek JB. 1981. Role of calcium in the hormonal regulation of liver metabolism. Biochem Biophys Acta 639:243–295.

Yamaguchi M. 2000a. Role of regucalcin in calcium signaling. Life Sci 66:1769–1780.

Yamaguchi M. 2000b. The role of regucalcin in nuclear regulation of regenerating liver. Biochem Biophys Res Commun 276:1–6.

Yamaguchi M, Isogai M. 1993. Tissue concentration of calcium binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay. Mol Cell Biochem 122:65–68.

Yamaguchi M, Katsumata T. 1999. Enhancement of protein kinase activity in the cytosol of regenerating rat liver. Regulatory role of endogenous regucalcin. Int J Mol Med 3:505–510.

Yamaguchi M, Ueoka S. 1997. Inhibitory effect of calcium-binding protein regucalcin on ribonucleic acid synthesis in isolated rat liver nuclei. Mol Cell Biochem 173:169–175.

Yamaguchi M, Yamamoto T. 1978. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull 26:1915–1918.

Yamaguchi M, Mori S, Kato S. 1988. Calcium-binding protein regucalcin is an activator of ($Ca^{2+}$–$Mg^{2+}$)-adenosine triphosphase in the plasma membranes of rat liver. Chem Pharm Bull 36:3532–3539.

Yamaguchi M, Morooka Y, Misawa H, Tsurusaki Y, Nakajima R. 2002. Role of endogenous regucalcin in transgenic rats: Suppression of kidney cortex cytosolic protein phosphatase activity and enhancement of heart muscle microsomal $Ca^{2+}$-ATPase activity. J Cell Biochem 86:520–529.