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Suppressive Effect of Endogenous Regucalcin on Nitric Oxide Synthase Activity in Cloned Rat Hepatoma H4-II-E Cells Overexpressing Regucalcin

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Abstract The role of endogenous regucalcin, which is a regulatory protein in calcium signaling, in the regulation of nitric oxide (NO) synthase activity in the cloned rat hepatoma H4-II-E cells was investigated. Hepatoma cells were cultured for 24–72 h in the presence of fetal bovine serum (FBS; 10%). NO synthase activity in the 5,500 *g* supernatant of cell homogenate was significantly increased by the addition of calcium chloride (10 μM) and calmodulin (2.5 μg/ml) in the enzyme reaction mixture. The presence of trifluoperazine (TFP; 50 μM), an antagonist of calmodulin, inhibited the effect of calcium (10 μM) addition in increasing NO synthase activity, indicating the existence of Ca^{2+}/calmodulin-dependent NO synthase in hepatoma cells. NO synthase activity was significantly decreased by the addition of regucalcin (10^{-8} or 10^{-7} M) in the reaction mixture without or with Ca^{2+}/calmodulin addition. The effect of regucalcin (10^{-7} M) in decreasing NO synthase activity was also seen in the presence of TFP (50 μM) or EGTA (1 mM). The presence of anti-regucalcin monoclonal antibody (10–50 ng/ml) in the reaction mixture caused a significant elevation of NO synthase activity. NO synthase activity was significantly suppressed in the hepatoma cells (transfectants) overexpressing regucalcin. This decrease was completely abolished in the presence of anti-regucalcin monoclonal antibody (50 ng/ml) in the reaction mixture. Moreover, the effect of Ca^{2+}/calmodulin addition in increasing NO synthase activity in the hepatoma cells (wild-type) was completely prevented in transfectants. The present study demonstrates that endogenous regucalcin has a suppressive effect on NO synthase activity in the cloned rat hepatoma H4-II-E cells. J. Cell. Biochem. 89: 800–807, 2003.

Key words: regucalcin; NO synthase; calcium signaling; calmodulin; hepatoma cells; transfectant

Calcium ion (Ca^{2+}) plays a pivotal role in the regulation of many cell functions. The Ca^{2+} effect in cells is amplified by calmodulin and 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; Misawa and Yamaguchi, 2000], has been demonstrated to play a multifunctional role as an inhibitory protein in Ca^{2+}-signaling process in cells [Yamaguchi, 2000a,b; reviews].

It has been shown that regucalcin 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, which regucalcin is predominantly expressed [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. Also, regucalcin has been demonstrated to have an inhibitory effect on protein kinases and protein phosphatases in the kidney cortex and brain of rats [Kurota and Yamaguchi, 1998; Hamamo and Yamagucih, 2001; Morooka and Yamaguchi, 2002]. Moreover, regucalcin has been shown to suppress overexpression of cell proliferation due to inhibiting deoxyribonucleic acid and ribonucleic acid syntheses in the nucleus of regenerating rat liver [Tsurusaki and Yamaguchi, 2002a,b]. Thus regucalcin may

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play a pivotal role 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, acts as a messenger or modulator molecule in many biological systems. NO is produced from L-arginine with L-citrullin as coproduced in a reaction catalyzed by NO synthase that requires NADPH, Ca^{2+}/calmodulin, and others [Lowenstein et al., 1994; Lee and Stull, 1998]. More recently, it has been shown that regucalcin can inhibit NO synthase activity in the cytoplasm of rat liver [Yamaguchi et al., 2003], suggesting a novel role of regucalcin in the regulation of NO-related cellular function.

The present study, furthermore, was undertaken to determine whether endogenous regucalcin has a suppressive effect on NO synthase activity in the cultured proliferative cells. It was found that endogenous regucalcin plays a suppressive role in the regulation of NO synthase activity in the cloned rat hepatoma H4-II-E cells.

MATERIALS AND METHODS

Chemicals

α-Minimun essential medium (α-MEM) and penicillin–streptomycin solution (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), arginine, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), citrulline, calmodulin (52,000 U/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.

Isolation of Regucalcin

Male Wistar rats, weighing 100–120 g, were obtained commercially from Japan SLC (Hamamatsu, Japan). Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. Regucalcin was isolated from rat liver cytosol. 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 (w/v) 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 recentrifuged 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, 1999]. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg/animal of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 days later antigen (0.25 mg/animal) was intraperitoneally injected with Freund's incomplete adjuvant. Animals were killed by bleeding 3 days after the last injection. Spleen cells were prepared from immunized 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).

Cell Culture

The cloned rat hepatoma H4-II-E cells and the transfectant of H4-II-E cells (1.0×10^5) were maintained for 24–72 h in α-MEM supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain confluent monolayers [Yamaguchi and Nakajima, 1999; Misawa et al., 2002]. After culture, cells were washed three times with phosphate-buffered saline (PBS), scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 s with an ultrasonic device. Scrapped cells were also homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were spun at 5,500 *g* in a refrigerated centrifuge for 5 min to remove nuclei and mitochondria. The

5,500*g* supernatant was pooled to assay NO synthase activity and to analyze regucalcin protein with Western blot. Protein concentration in the 5,500*g* supernatant of cell homogenate was determined by the method of Lowry et al. [1951].

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 a *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 pEXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as regucalcin (RC)/pCXN2 [Misawa et al., 2002].

For transient transfection assay, the H4-II-E cells were grown on 35-mm dishes to approximately 70% confluence. Each of RC/pCXN2 and pCXN2 vector alone was transfected into H4-II-E 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. H4-II-E 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 α-MEM containing 10% FBS.

Determination of Cell Numbers

After trypsinization using 0.2% trypsin plus 0.02% ethylenediamine-tetraacetic acid in Ca^{2+}/Mg^{2+}-free PBS, cell numbers were determined by electronic particle counter.

Assay of NO Synthase

NO synthase activity in the 5,500*g* supernatant of the cloned rat hepatoma 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 (50–80 μg/ml) in the absence or presence of calmodulin (2.5 μg/ml). In separate experiments, the reaction mixture contained either vehicle, calcium chloride (10 μM), regucalcin (10^{-9}–10^{-7} M), 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 (min) per milligram (mg) of cell protein.

Western Blot Analysis

The homogenate from the cloned rat hepatoma cells and transfectants cultured with 10% FBS was centrifuged for 10 min at 5,500*g* at 4°C, and the supernatant was used for Western blot analysis [Wessendorf et al., 1993]. Aliquots of protein (20 μg) were mixed with 5 × Laemmli 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-regucalcin 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) Tween 20 (washing buffer), and 5% (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 again they were washed. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacture's instruction. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

Statistical Analysis

Data were expressed as the mean ± SEM. The significance of difference between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. A *P*-value of <0.01 was considered to indicate statistically significant difference.

RESULTS

Effect of Regucalcin on NO Synthase Activity in Hepatoma Cells

The cloned rat hepatomaH4-II-E cells were cultured for 24, 48, or 72 h in culture medium containing 10% FBS. NO synthase activity in the cells was measured in the reaction mixture containing either vehicle, calcium chloride (10 μM) or calcium chloride (10 μM) plus calmodulin (2.5 μg/ml) (Fig. 1). NO synthase activity was not significantly altered with culture for 24–72 h. The addition of calcium in the enzyme reaction mixture did not have a significant effect on NO synthase activity in the cells obtained with 24 h- or 48 h-culture. However, the enzyme activity was significantly increased by the addition of calcium (10 μM) plus calmodulin (2.5 μg/ml). In the cells with 72 h-culture, NO synthase activity was significantly elevated by the addition of calcium (10 μM) or calcium (10 μM) plus calmodulin (2.5 μg/ml) in the reaction mixture.

The effect of TFP, an antagonist of calmodulin [Vincenzi, 1982], on NO synthase activity in the cells cultured for 24–72 h in culture medium containing 10% FBS was examined (Fig. 2). The presence of TFP (50 μM) in the enzyme reaction mixture containing either vehicle or calcium chloride (10 μM) caused a significant decrease in NO synthase activity. Thus Ca^{2+}/calmodulin-dependent NO synthase activity was present in the cloned rat hepatoma H4-II-E cells.

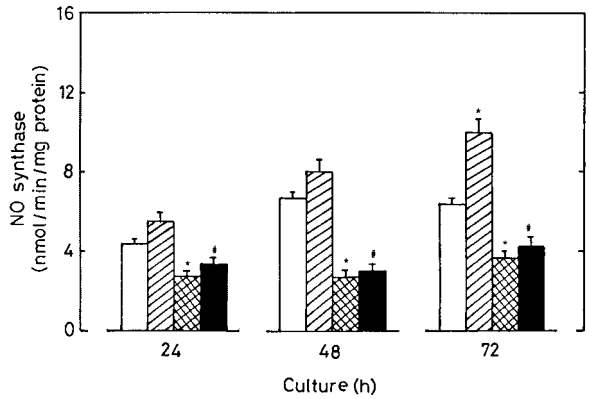

Fig. 2. Effect of trifluoperazine (TFP) on NO synthase activity in the cloned rat hepatoma H4-II-E cells cultured with 10% FBS. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle, calcium chloride (10 μM), TFP (50 μM), or calcium chloride (10 μM) plus TFP (50 μM). Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value obtained from calcium addition. White bars, control; hatched bars, calcium; double hatched bars, TFP; black bars, calcium plus TFP.

The effect of regucalcin on NO synthase activity in the hepatoma cells is shown in Figure 3. Cells were cultured for 72 h in medium containing 10% FBS. NO synthase activity was measured in the reaction mixture containing either vehicle or calcium chloride (10 μM) plus

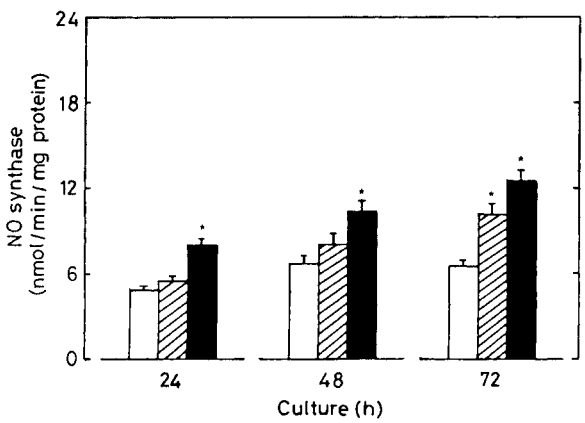

Fig. 1. Change in NO synthase activity in the cloned rat hepatoma H4-II-E cells cultured with 10% FBS. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle, calcium chloride (10 μM) or calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, calcium addition; black bars, calcium plus calmodulin addition.

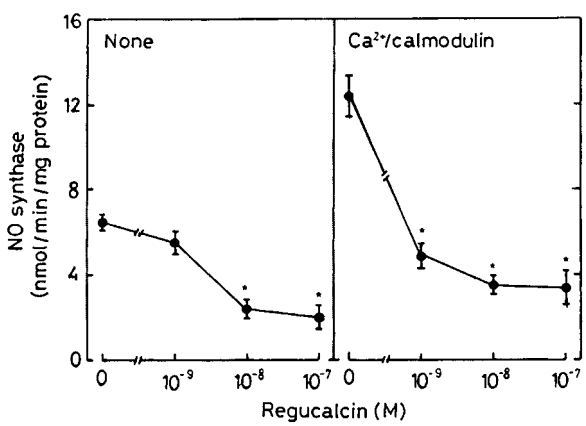

Fig. 3. Effect of regucalcin on NO synthase activity in the cloned rat hepatoma H4-II-E cells cultured with 10% FBS. Cells were cultured for 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle or regucalcin (10^{-9}–10^{-7} M) in the absence or presence of calcium chloride (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 regucalcin addition.

calmodulin (2.5 μg/ml). The addition of regucalcin (10^{-9}–10^{-7} M) in the reaction mixture caused a significant decrease in Ca^{2+}/calmodulin-increased NO synthase activity. The inhibitory effect of regucalcin (10^{-8} and 10^{-7} M) was also seen in the reaction mixture without Ca^{2+}/calmodulin addition. The effect of regucalcin (10^{-7} M) in decreasing NO synthase activity of the cells cultured for 72 h was also seen in the reaction mixture containing either TFP (50 μM) or EGTA (1 mM) without Ca^{2+}/calmodulin addition (Fig. 4).

Effect of Anti-Regucalcin Antibody on NO Synthase Activity in Hepatoma Cells

The role of endogenous regucalcin in the regulation of NO synthase activity in the cloned rat hepatoma H4-II-E cells was examined. Cells were cultured for 24–72 h in medium containing 10% FBS. NO synthase activity was measured in the reaction mixture without Ca^{2+}/calmodulin addition. The presence of anti-regucalcin monoclonal antibody (10, 20, or 50 ng/ml) in the reaction mixture caused a significant increase in NO synthase activity in the cells cultured for 24, 48, or 72 h (Fig. 5). The effect of antibody (20 or 50 ng/ml) was remarkable in the cells cultured for 48 h.

The expression of regucalcin in the cloned rat hepatoma H4-II-E cells has been shown to be stimulated by culture with 10% FBS as compared with that of 1% FBS [Yamaguchi and Nakajima, 1999; Inagaki and Yamaguchi, 2001b]. The change in NO synthase activity in the cells cultured with 1 or 10% FBS for 24–72 h was examined (Fig. 6). NO synthase activity was markedly increased by culture with 10% FBS. The effect of anti-regucalcin monoclonal antibody (50 ng/ml) in increasing NO synthase activity in the reaction mixture without Ca^{2+}/calmodulin addition was significantly enhanced in the cells cultured with 10% FBS as compared with that of 1% FBS.

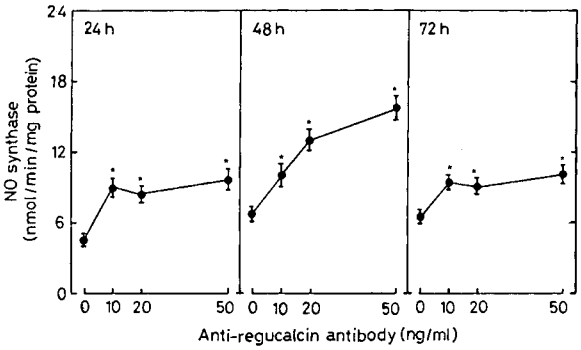

Fig. 5. Effect of anti-regucalcin monoclonal antibody on NO synthase activity in the cloned rat hepatoma H4-II-E cells cultured with 10% FBS. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle or anti-regucalcin monoclonal antibody (10, 20, or 50 ng/ml) without calcium and calmodulin. Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compared with the control (none).

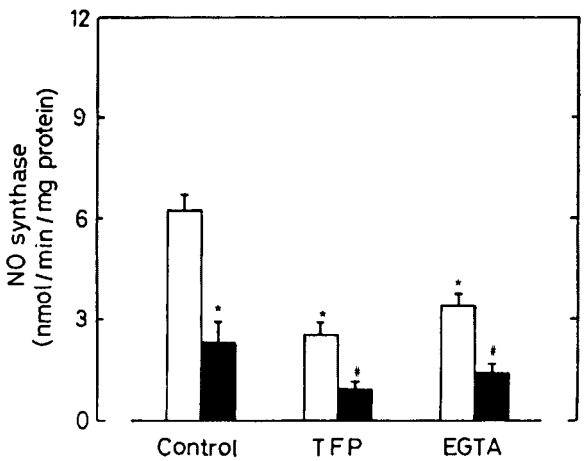

Fig. 4. Effect of regucalcin on TFP- or EGTA-decreased NO synthase activity in the cloned rat hepatoma H4-II-E cells cultured with 10% FBS. The enzyme reaction mixture contained either vehicle, TFP (50 μM) or EGTA (1 mM) in the absence or presence of regucalcin (10^{-7} M). Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value from TFP or EGTA alone. White bars, control; black bars, regucalcin.

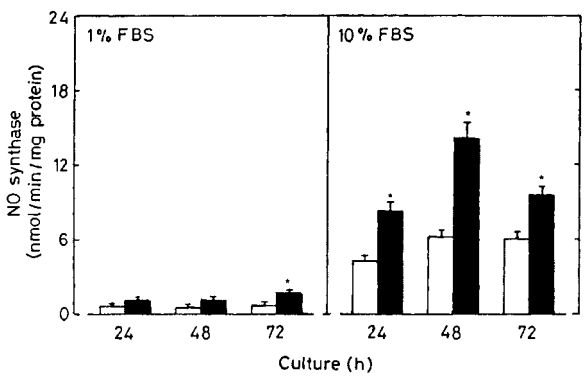

Fig. 6. Effect of anti-regucalcin monoclonal antibody on NO synthase activity in the cloned rat hepatoma H4-II-E cells cultured with 1 or 10% FBS. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle or anti-regucalcin monoclonal antibody (50 ng/ml). $^{*}P < 0.01$, compared with the control (none) value. White bars, control; black bars, antibody.

Change in NO Synthase Activity in Hepatoma Cells Overexpressing Regucalcin

The change in NO synthase activity in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin was examined. The hepatoma cells (wild-type), pCXN-2 transfected cells (mock-type), or stable regucalcin/pCXN-2 transfectants were cultured for 24–72 h in medium containing 10% FBS. The expression of regucalcin in the cells was remarkable in transfectants cultured for 24, 48, or 72 h (Fig. 7). NO synthase activity was not significantly altered in mock-type cells as compared with that of wild-type cells (Fig. 8). However, the enzyme activity was significantly lowered in transfectants. This decrease was seen with culture for 24, 48, or 72 h. The suppression of NO synthase activity in transfectants was completely abolished by the addition of anti-regucalcin monoclonal antibody (50 ng/ml) into the enzyme reaction mixture (Fig. 8).

The effect of Ca^{2+}/calmodulin addition on NO synthase activity in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin is shown in Figure 9. The addition of calcium chloride (10 μM) plus calmodulin (2.5 μg/ml) into the enzyme reaction mixture caused a remarkable increase in NO synthase activity in wild-type cells cultured for 24, 48, or 72 h. The effect of Ca^{2+}/calmodulin in increasing NO synthase activity was not seen in transfectants overexpressing regucalcin.

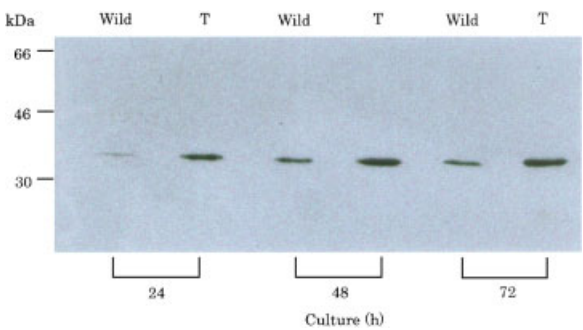

Fig. 7. Expression of regucalcin (RC) in the cloned rat hepatoma H4-II-E cells (wild-type), pCXN-2 transfected cells (mock-type) on stauble RC/pCXN2 transfectants. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. Western blot analysis was carried out on the extracts (15 μg of the 5,500 *g* supernatant of cell homogenate) obtained from the hepatoma cells. The figure shown is representative of four experiments. The densitometric data for regucalcin band in transfectants (T) cultured with 24, 48, or 72 h was 374 ± 6.5, 152 ± 5.1, or 155 ± 7.8 (% of wild-type; mean ± SEM for four experiments), respectively. Regucalcin levels at 24, 48, or 72 h of culture were significantly ($P < 0.01$) increased.

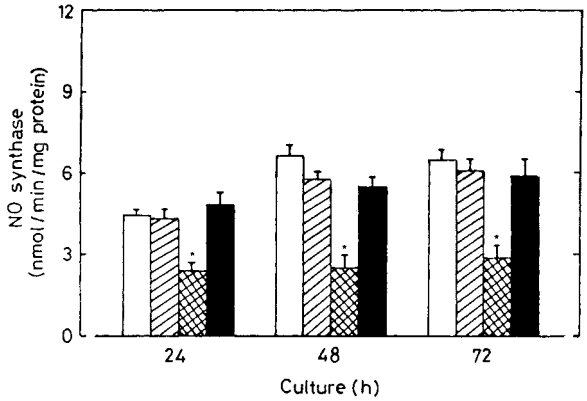

Fig. 8. Change in NO synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type), pCXN-2 transfected cells (mock-type), or stable RC/pCXN2 transfectant. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle or anti-regucalcin monoclonal antibody (50 ng/ml). Each value is the mean ± SEM of six experiments. *$P < 0.01$, compared with the control (none) value obtained from wild-type cells. White bars, wild-type cells; hatched bars, mock-type cells; double hatched bars, transfectant; black bars, antibody addition in transfectant.

DISCUSSION

The role of regucalcin in proliferative cells has been shown in the cloned rat hepatoma H4-II-E cells. Regucalcin has a suppressive effect on the enhancement of protein kinase activity [Inagaki and Yamaguchi, 2001a], protein tyrosine phosphatase activity [Inagaki and Yamaguchi, 2000], and deoxyribonucleic acid (DNA) synthesis activity [Inagaki and Yamaguchi, 2001b] with proliferation of the

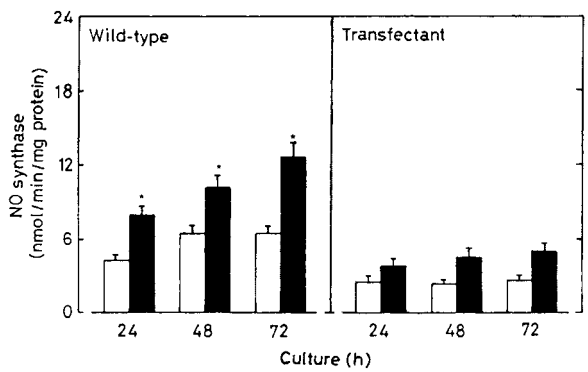

Fig. 9. Effect of calcium and calmodulin addition on NO synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type) or stable RC/pCXN2 transfectant. Cells were cultured for 24, 48, and 72 h in the presence of 10% FBS. The enzyme reaction mixture contained either vehicle or calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of six experiments. *$P < 0.01$, compared with the control (none) value. White bars, control; black bars, calcium plus calmodulin addition.

cloned rat hepatoma H4-II-E cells. Regucalcin has also been shown to suppress cell proliferation and DNA synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin [Misawa et al., 2002]. Regucalcin may have a suppressive effect on proliferation of the cloned rat hepatoma H4-II-E cells due to inhibiting the activity of protein kinase and protein tyrosine phosphatase, which is involved in signaling mechanism [Hunter, 1995].

The present study, furthermore, demonstrates that NO synthase activity is inhibited by endogenous regucalcin in the cloned rat hepatoma H4-II-E cells. NO, which is produced by NO synthase, acts as a messenger or modulator molecule in many biological systems [Lowenstein et al., 1994]. Regucalcin may act as a suppressor in the regulation of NO synthase activity in proliferative cells.

Ca^{2+}/calmodulin-dependent NO synthase was present in the cloned rat hepatoma H4-II-E cells. Regucalcin inhibited Ca^{2+}/calmodulin-dependent NO synthase activity. Moreover, the effect of regucalcin in decreasing NO synthase activity was found in the presence of TFP, an antagonist of calmodulin [Vincenzi, 1982], and EGTA, a chelator of Ca^{2+}, in the enzyme reaction mixture. Regucalcin has been shown to bind to calmodulin [Omura and Yamaguchi, 1998]. Presumably, regucalcin reveals an inhibitory effect on NO synthase activity due to binding to calmodulin and/or the enzyme in proliferative cells.

The presence of anti-regucalcin monoclonal antibody in the reaction mixture caused a significant increase in NO synthase activity in the cloned hepatoma H4-II-E cells, suggesting that endogenous regucalcin suppresses the enzyme activity in the cells. NO synthase activity was significantly enhanced in the hepatoma cells cultured with 10% FBS as compared with that of 1% FBS. The proliferation of hepatoma cells was markedly stimulated by culture with 10% FBS [Inagaki and Yamaguchi, 2000]. NO synthase activity was significantly elevated by culture with 10% FBS as compared with that of 1% FBS, suggesting that the enzyme is induced in proliferative cells. Moreover, the effect of anti-regucalcin monoclonal antibody in increasing NO synthase activity was significantly enhanced in the cloned hepatoma cells cultured with 10% FBS as compared with that of 1% FBS. Regucalcin levels were elevated in the cloned rat hepatoma cells with 10% FBS-culture [Yamaguchi and Nakajima, 1999; Inagaki and Yamaguchi, 2001b]. Endogenous regucalcin may suppress the enhancement of NO synthase activity with proliferation of hepatoma cells.

NO synthase activity was found to decrease markedly in the cloned hepatoma cells overexpressing regucalcin. This decrease was completely abolished in the presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture. Moreover, the effect of Ca^{2+}/calmodulin addition in increasing NO synthase activity in hepatoma cells (wild-type) was not seen in the transfectants. These findings further support the view that regucalcin suppresses NO synthase activity in the cloned hepatoma cells.

Whether endogenous regucalcin suppresses NO production in the cloned rat hepatoma H4-II-E cells is unknown at present. However, it is speculated that regucalcin may inhibit NO production in hepatoma cells, since the protein can decrease NO synthase activity in the cells. A high concentration of NO, which is produced from inducible NO synthase, has been shown to inhibit cell proliferation [Hukkanen et al., 1995; Costa and Assreuy, 2002] and to induce apoptosis of hepatoma cells [Liu et al., 2000; Mozart et al., 2001]. Meanwhile, it is reported that a low concentration of NO, which is produced from endothelial NO synthase, protects against the cytotoxic effects of reactive oxygen species in cells [Wink et al., 1994]. It is possible that endogenous regucalcin may have an inhibitory effect on inducible and endothelial NO synthases in hepatoma cells. Alternatively, regucalcin may have a physiologic role in the regulation of NO-related cell functions.

In conclusion, it has been demonstrated that endogenous regucalcin has a suppressive effect on the enhancement of NO synthase activity with proliferation of the cloned rat hepatoma H4-II-E cells.

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