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Overexpression of Regucalcin Enhances Glucose Utilization and Lipid Production in Cloned Rat Hepatoma H4-II-E Cells: Involvement of Insulin Resistance

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Abstract The role of regucalcin, which is a regulatory protein in intracellular signaling pathway, in the regulation of glucose utilization and lipid production was investigated using the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. The hepatoma cells (wild-type) and stable regucalcin/pCXN2-transfected cells (transfectant) were cultured for 72 h in a medium containing 10% fetal bovine serum (FBS) to obtain subconfluent monolayers. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} or 10^{-7} M) with or without supplementation of glucose (10, 25, or 50 mg/ml of medium) in the absence of insulin. The production of triglyceride and free fatty acid was significantly increased in transfectants cultured without insulin and glucose supplementation as compared with that of wild-type cells. The supplementation of glucose (10, 25, or 50 mg/ml) caused a remarkable increase in medium glucose consumption, triglyceride, and free fatty acid productions in transfectants cultured without insulin. The presence of insulin (10^{-7} M) caused a significant increase in medium glucose consumption, triglyceride, and free fatty acid productions in wild-type cells cultured with glucose supplementation. These increases were significantly prevented in transfectants cultured for 72 h. The expression of acetyl-CoA carboxylase, HMG-CoA reductase, glucokinase, pyruvate kinase, and glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) mRNAs in wild-type cells was not significantly changed by culture with or without glucose supplementation in the presence of insulin. These gene expressions were not significantly changed in transfectants. The expression of glucose transporter 2 mRNA was significantly increased in transfectants as compared with that of wild-type cells. Such an increase was not seen in transfectants cultured in the presence of insulin with or without glucose supplementation. This study demonstrates that overexpression of regucalcin enhances glucose utilization and lipid production in the cloned rat hepatoma H4-II-E cells, and that it regulates the effect of insulin. J. Cell. Biochem. 99: 1582–1592, 2006.

Key words: regucalcin; glucose metabolism; lipid metabolism; insulin; cell signaling; liver cells

Regucalcin was found as a novel Ca^{2+}-binding protein not including the EF-hand motif [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988; Shimokawa and Yamaguchi, 1993]. The name regucalcin was proposed for this Ca^{2+}-binding protein, which can regulate the Ca^{2+} and/or calmodulin effects on various enzymes in liver cells [Yamaguchi and Mori, 1988; Yamaguchi, 1992 in reviews]. Regucalcin has been demonstrated to play a multifunctional role as a regulatory protein in intracellular signaling process in many cell types [Yamaguchi, 2000a,b, 2005; review] in recent years.

The gene of regucalcin is highly conserved in vertebrate species [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The rat and human regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. Regucalcin messenger ribonucleic acid (mRNA) and its

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protein are greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999]. AP-1, NF1-A1, and RGPR-p117 (novel protein) have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1999; Misawa and Yamaguchi, 2001, 2002; Sawada and Yamaguchi, 2006].

Regucalcin plays a role in the maintenance of intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinases, tyrosine kinases, protein phosphatases, nitric oxide (NO) synthase, cytosolic protein synthesis, nuclear DNA and RNA syntheses in many cell types [Yamaguchi, 2000a,b, 2005], and it has an activatory effect on Ca^{2+}-ATPase (calcium pump enzymes), proteolysis (protease), and superoxide dismutase in cells [Yamaguchi and Tai, 1992; Fukaya and Yamaguchi, 2004; Yamaguchi, 2005]. Regucalcin has been shown to have suppressive effects on cell proliferation [Nakagawa et al., 2005; Yamaguchi and Daimon, 2005] and tumor necrosis factor-α, lipopolysaccharide-, or thapsigargin-induced apoptotic cell death [Izumi and Yamaguchi, 2004; Nakagawa and Yamaguchi, 2005]. Thus, regucalcin plays a pivotal role in maintaining cell homeostasis and function [Yamaguchi, 2005 in review].

Regucalcin transgenic rats have been shown to induce bone loss [Yamaguchi et al., 2002] and hyperlipidemia [Yamaguchi et al., 2004a], suggesting its pathophysiologic role. The regucalcin transgenic rats-induced bone loss is involved in the mechanism by which the protein has activatory effects on osteoclastic bone resorption [Yamaguchi et al., 2004b; Yamaguchi and Uchiyama, 2005] and suppressive effects on cell differentiation and mineralization in osteoblastic cells in vitro [Yamaguchi et al., 2005]. Whether regucalcin regulates lipid metabolism in liver cells is unknown, however.

This study was undertaken to determine whether overexpression of regucalcin can stimulate the production of lipids in the cloned rat hepatoma H4-II-E cells in vitro. We found that overexpressing of regucalcin enhances glucose utilization and lipid production in the H4-II-E cells.

MATERIALS AND METHODS

Chemicals

α-Minimal essential medium (α-MEM) and penicillin–streptomycin (5,000 U/ml penicillin: 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) and insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Glucose and other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). Reagents used were dissolved in distilled water, and some reagents were passed through ion-exchange resin to remove metal ions.

Regucalcin Transfectants

Regucalcin transfectants, which are overexpressing regucalcin in the cloned rat hepatoma H4-II-E cells, were used in this experiments as reported previously [Misawa et al., 2002]. The cDNA encoding rat regucalcin was isolated and cloned into the pBluscript vector [Shimokawa and Yamaguchi, 1993]. The regucalcin cDNA contains *P*st 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 pCXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as regucalcin/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 regucalcin/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, Madison WI). 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 cloned were isolated, transferred to 35-mm dishes, and grown in the medium without neomysin. We confirmed that regucalcin was stably expressed in the transfectants using Western blot analysis for regucalcin protein levels [Misawa et al., 2002]. In experiments, transfectants were cultured for 72 h in α-MEM containing 10% FBS.

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 72 h in α-MEM supplemented with 50 U/ml penicillin and 50 μg/ml streptomysin in humidified 5% CO_2/95% air at 37°C to obtain subconfluent monolayers [Misawa et al., 2002]. In experiments, cells were cultured for 24–72 h in medium containing either vehicle or insulin (10^{-8} M) with or without glucose supplementation in the absence of FBS. After culture, the medium was pooled, and then cells were washed three times with phosphate-buffered saline (PBS), and collected to determine cellular DNA.

Biochemical Assay in Culture Medium

After culture, the medium was collected and stored at −80°C until assay. Glucose, triglyceride, and free fatty acid concentrations in the medium were determined using the assay kit (Wako Pure Chemical Co.). The consumption of medium glucose and the production of triglyceride or free fatty acid in H4-II-E cells (wild-type) and transfectants were expressed as milligram or microequivalent (μEq) per milligrams of cellular DNA, respectively.

Cellular DNA Assay

To measure the DNA content in H4-II-E cells (wild-type) and transfectants, the cultured cells were scraped by addition of 0.5 ml of ice-cold 0.1N NaOH solution and disrupted for 30 s with an ultrasonic device. After alkali extraction, DNA content in the extract was determined using the method of Ceriotti [1951].

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. After culture, cells were washed three times with ice-cold PBS, and then cells were homogenized in buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isoprepanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethyl-pyrocarbonate-treated water.

RT-PCR was preformed with a Titam™ One Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. Primers for amplification of rat acetyl-coenzyme A (acetyl-CoA) carboxylase cDNA were: 5′-CCTCGGCACATGGAGATGTA-3′ (sense strand, positions 6301–6320 of cDNA sequence) and 5′-CCGCTCCTTCAACTTG CTCT-3′ (antisense strand, positions 6491–6510) [Lopez-Casillas et al., 1988]. The pair of oligonucleotide primers were designed to amplify a 210-bp sequence from the mRNA of rat acetyl-CoA corboxylase. Primers for amplification of rat 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase cDNA were: 5′-GGCGTGCAAA-GACAATCCTG-3′ (sense strand, positions 2522–2541 of cDNA sequence) and 5′-TGCC CGTGTTTCAGTCCAGT-3′ (antisense strand, positions 2727–2746) [Cemielewski et al., 2003]. The pair of oligonucleotide primers were designed to amplify a 225-bp sequence from the mRNA of rat HMG-CoA reductase. Primers for amplication of rat glucokinase cDNA were: 5′-GCACT GCCGAGATGCTCTTT-3′ (sense strand, positions 441–460 of cDNA sequence) and 5′-CAT TGTGGCCACTGTGTCGT-3′ (antisense strand, positions 702–721) [Anderson et al., 1989]. The pair of oligonucleotide primers was designed to amplify a 281-bp sequence from the mRNA of rat glucokinase. Primers for amplication of rat pyruvate kinase cDNA were: 5′-TGGTATCTTGGGCAGCAG GA-3′ (sense strand, positions 652–671 of cDNA sequence) and 5′-CAGGGATCTCAATGCCCAG-GTCAC-3′ (antisense strand, positions 936–959) [Inoue et al., 1986]. The pair of oligonucleotide primers was designed to amplify a 308-bp sequence from the mRNA of rat pyruvate kinase. Primers for amplication of rat glucose transporter 2 cDNA were: 5′-TTCATGTCGCTGGGACTGGT-3′ (sense strand, positions 1246–1265 of cDNA sequence) and 5′-CCACCCCAGCAAAAAGGA-AG-3′ (antisense strand, positions 1500–1519) [Thorens et al., 1988]. The pair of oligonucleotide primers were designed to amplify a 274-bp sequence from the mRNA of rat glucose transporter 2. For semiquantitative PCR, G3PDH was used as an internal control to evaluate total RNA input. Primers for amplification of G3PDH cDHA were, 5′-GATTTGGCCGTATCGGAC GC-3′ (sense strand) and 5′-CTCCTTGGAGGCC-ATGTAGG-3′ (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977-bp sequence from the mRNA of rat G3PDH.

RT-PCR was performed using reaction mixture (20 μl) containing 2 μg of total RNAs, supplied RT-TCR buffer, Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 μM primers. Samples were

incubated at 50°C for 30 min, and then amplified for 30 cycles under the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 56°C, and extension for 60 s at 62.0°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Image density was quantified with a FluoroImager SI (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. *P*-values less than 0.05 were considered to indicate statistically significant differences. Also, we used an ANOVA multiple comparison test to compare the treatment groups.

RESULTS

Lipid Production in H4-II-E Cells Overexpressing Regucalcin

The cloned rat hepatoma H4-II-E cells (wild-type) or stable regucalcin/pCXN2-transfected cells (transfectants) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers. Cells with subconfluency were further cultured for 24 or 72 h in a medium without FBS. The consumption of medium glucose was not significantly changed in transfectants as compared with that of wild-type cells (Fig. 1A). The production of triglyceride and free fatty acid was significantly increased in transfectants with culture for 72 h (Fig. 1B,C). A significant increase in triglyceride production was also seen with culture for 24 h (Fig. 1B).

Effect of Glucose Supplementation on Lipid Production in H4-II-E Cells Overexpressing Regucalcin

Wild-type cells or transfectants with subconfluency were culture for 72 h in medium supplemented with glucose (10, 25, or 50 mg/ml of medium) without FBS. The consumption of medium glucose in wild-type cells was significantly increased with supplementation of glucose (10, 25, or 50 mg/ml) (Fig. 2). This increase was significantly enhanced in transfectants. The production of triglyceride in wild-type cells was significantly increased with supplementation of glucose (10, 25, or

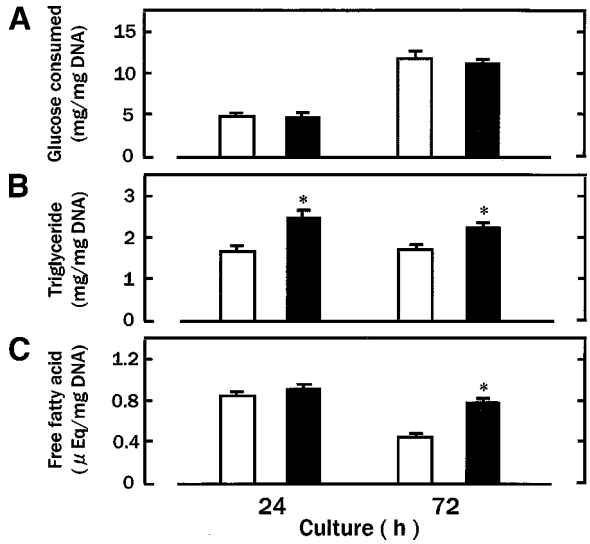

Fig. 1. The change in medium glucose consumption, triglyceride, and free fatty acid productions in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing FBS (10%) to obtain subconfluent monolayers. After medium change, cells with subconflency were cultured for 24 or 72 h in medium without BS. At each culture time, the medium and cells were collected for assay. Each value is the mean ± SEM of six experiments with separate culture. *$P < 0.01$ compared with the control value obtained from wild-type cells. Open bars, wild-type cells: Closed bars, transfectants.

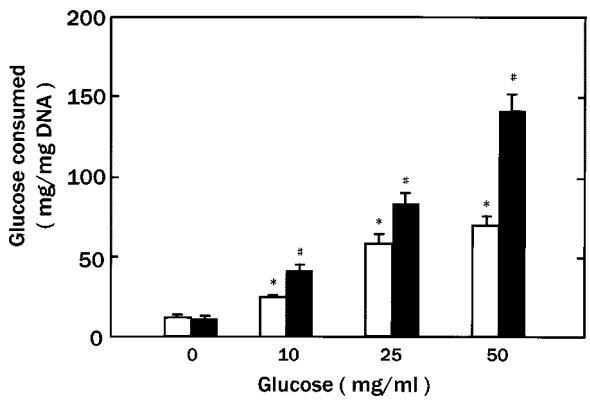

Fig. 2. Change in medium glucose consumption in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells with supplementation of glucose. Cells with subconfluency were cultured for 72 h in medium with supplementation of glucose (0, 10, 25, or 50 mg/ml of medium). After culture, the medium and cells were collected to assay. Each value is the mean ± SEM of six experiments with separate culture. *$P < 0.01$ compared with the value without glucose supplementation. #$P < 0.01$ compared with the control value obtained from wild-type cells with glucose supplementation. White bars, wild-type cells: black bars, transfectants.

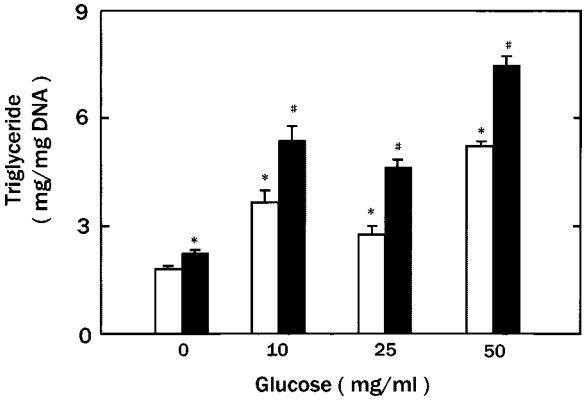

Fig. 3. Change in triglyceride production in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells with supplementation of glucose. Cells with subconfluency were cultured for 72 h in medium with supplementation of glucose (0, 10, 25, or 50 mg/ml of medium). After culture, the medium and cells were collected to assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control value without glucose supplementation. $^{\#}P < 0.01$ compared with the value obtained from wild-type cells with glucose supplementation. White bars, wild-type cells: black bars, transfectants.

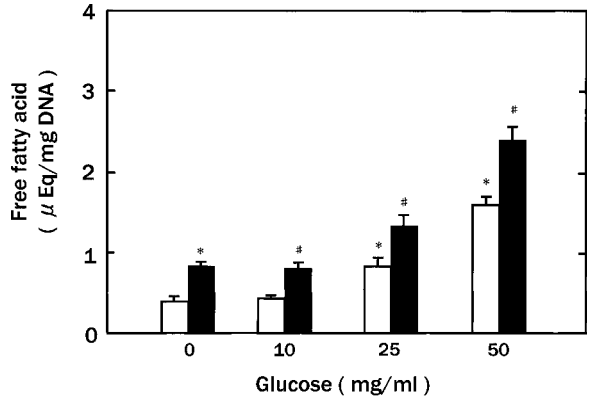

Fig. 4. Change in free fatty acid production in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells with supplementation of glucose. Cells with subconfluency were cultured for 72 h in medium with supplementation of glucose (0, 10, 25, or 50 mg/ml of medium). After culture, the medium and cells were collected for assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control value without glucose supplementation. $^{\#}P < 0.01$ compared with the value obtained from wild-type cells with glucose supplementation. White bars, wild-type cells: black bars, transfectants.

50 mg/ml) (Fig. 3). This increase was significantly enhanced in transfectants. The production of free fatty acid in wild-type cells was significantly increased with supplementation of glucose (25 or 50 mg/ml) (Fig. 4). This increase was significantly enhanced in transfectants.

Effects of Insulin on Lipid Production in H4-II-E Cells Overexpressing Regucalcin

Wild-type cells or transfectants with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} or 10^{-7} M) without FBS and glucose supplementation. Medium glucose consumption was significantly decreased in wide-type cells cultured in the presence of insulin (10^{-7} M) for 24 or 72 h (Fig. 5). Such an effect was also seen in wild-type cells cultured for 72 h in the presence of insulin (10^{-7} M). The effect of insulin in decreasing medium glucose consumption was significantly enhanced in transfectants cultured for 24 or 72 h (Fig. 5). Overexpressing of regucalcin enhanced the effect of insulin on medium glucose consumption.

The production of triglyceride in wild-type cells was significantly increased with culture for 24 h in the presence of insulin (10^{-8} M) (Fig. 6). Such an increase was not seen with insulin (10^{-7} M). The effect of insulin was not changed in transfectants. Culture with insulin (10^{-8} or 10^{-7} M) for 72 h caused a significant increase in the production of triglyceride in wild-type cells and transfectants (Fig. 6). The production

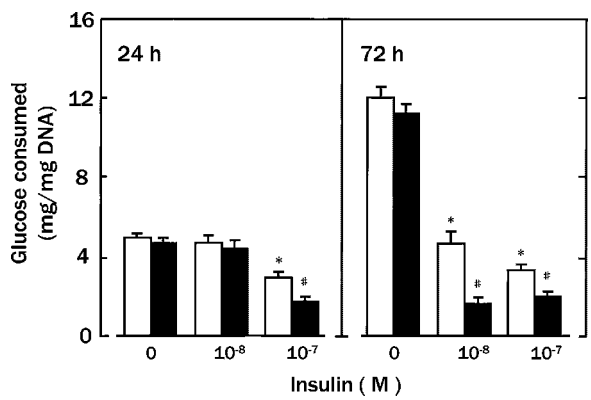

Fig. 5. Effect of insulin on medium glucose consumption in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M). At each culture time, the medium and cells were collected for assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control (none) value of wild-type cells. $^{\#}P < 0.01$ compared with the value of wild-type cells cultured with insulin. White bars, wild-type cells: black bars, transfectants.

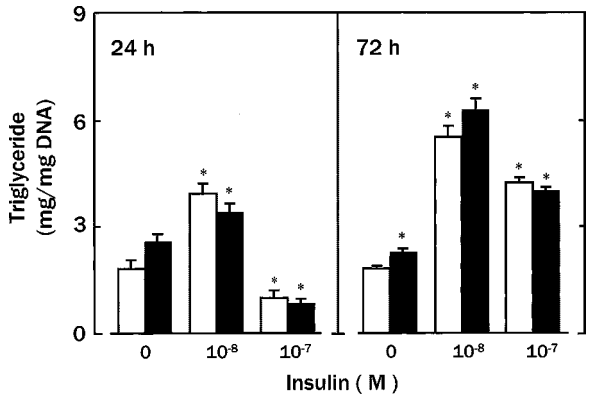

Fig. 6. Effect of insulin on triglyceride production in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M). At each culture time, the medium and cells were collected to assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control (none) value of wild-type cells. $^{\#}P < 0.01$ compared with the value of wild-type cells cultured with insulin. White bars, wild-type cells: black bars, transfectants.

of free fatty acid in wild-type cells was significantly increased with culture for 72 h in the presence of insulin (10^{-8} or 10^{-7} M) (Fig. 7). The effect of insulin (10^{-7} M) on free fatty acid production was significantly weakened in transfectants cultured for 72 h.

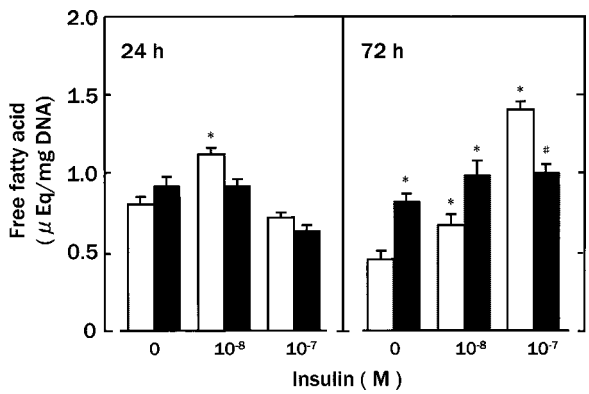

Fig. 7. Effect of insulin on free fatty acid production in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M). At each culture time, the medium and cells were collected to assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control (none) value of wild-type cells. $^{\#}P < 0.01$ compared with the value of wild-type cells cultured with insulin. White bars, wild-type cells: black bars, transfectants.

Effects of Insulin on Lipid Production in H4-II-E Cells Overexpressing Regucalcin With Supplementation of Glucose

Wild-type cells or transfectants with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} or 10^{-7} M) with the supplementation of glucose (50 mg/ml of medium) in the absence of FBS. The consumption of medium glucose in wild-type cells cultured with glucose supplementation was significantly increased by culture with insulin (10^{-8} M) for 24 or 72 h (Fig. 8). Culture with insulin (10^{-7} M) caused a significant increase in glucose consumption in wild-type cells. These increases were significantly inhibited in transfectants.

The production of triglyceride in wild-type cells cultured with glucose supplementation was significantly increased by culture with insulin (10^{-8} M) for 24 or 72 h (Fig. 9). Culture with insulin (10^{-7} M) for 72 h caused a significant increase in triglyceride production in wild-type cells.

The production of free fatty acid in wild-type cells cultured with glucose supplementation was significantly increased by culture with insulin (10^{-8} or 10^{-7} M) for 24 h (Fig. 10). Culture with insulin (10^{-7} M) for 72 h caused a

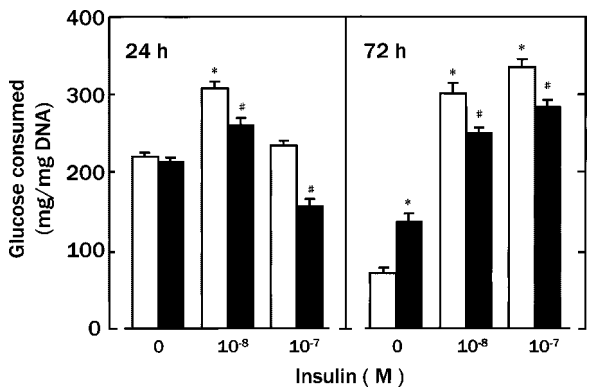

Fig. 8. Effect of insulin on medium glucose consumption in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells cultured with supplementation of glucose. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M) with supplementation of glucose (50 mg/ml of medium). At each culture time, the medium and cells were collected for assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control (none) value of wild-type cells cultured with glucose supplementation. $^{\#}P < 0.01$ compared with the value of wild-type cells cultured with insulin and glucose supplementation. White bars, wild-type cells: black bars, transfectants.

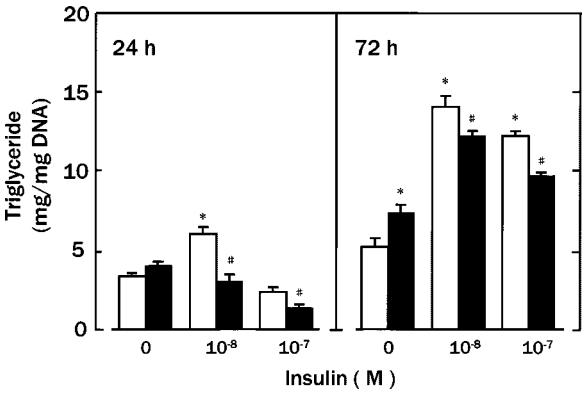

Fig. 9. Effect of insulin on triglyceride production in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells cultured with supplementation of glucose. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M) with supplementation of glucose (50 mg/ml of medium). At each culture time, the medium and cells were collected for assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control (none) value of wild-type cells cultured with glucose supplementation. $^{\#}P < 0.01$ compared with the value of wile-type cells cultured with insulin and glucose supplementation. White bars, wild-type cells: black bars, transfectants.

significant increase in free fatty acid production in wild-type cells. The effect of insulin (10^{-7} M) in increasing free fatty acid production was significantly inhibited in transfectants cultured for 72 h.

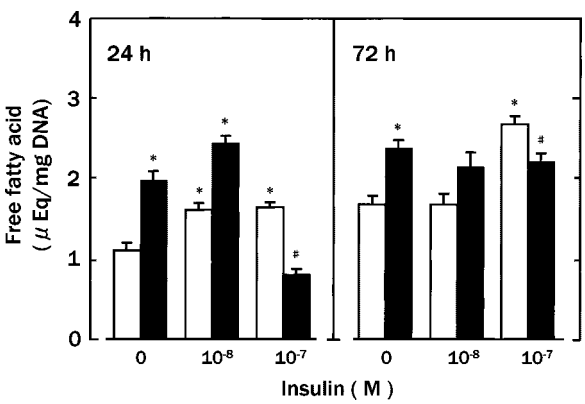

Fig. 10. Effect of insulin on free fatty acid production in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells cultured with supplementation of glucose. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M) with supplementation of glucose (50 mg/ml of medium). At each culture time, the medium and cells were collected to assay. Each value is the mean ± SEM of six experiments with separate culture. $^{*}P < 0.01$ compared with the control (none) value of wild-type cells cultured with glucose supplementation. $^{\#}P < 0.01$ compared with the value of wild-type cells cultured with insulin and glucose supplementation. White bars, wild-type cells: black bars, transfectants.

Gene Expression of Glucose and Lipid Metabolisms-Related Proteins in H4-II-E Cells Overexpressing Regucalcin

Wild-type cells or transfectants with subconfluency were cultured for 24 or 48 h in medium containing either vehicle or insulin (10^{-7} M) with or without supplementation of glucose (50 mg/ml of medium). The expression of acetyl-CoA carboxylase, HMG-CoA reductase, glucokinase, pyruvate kinase, glucose transporter 2, and G3PDH mRNAs in the cells was examined using RT-PCR analysis. The expression of acetyl-CoA carboxylase (Fig. 11A), HMG-CoA reductase (Fig. 11B), glucokinase (Fig. 12A), pyruvate kinase (Fig. 12B), and G3PDH (Fig. 13B) mRNAs in wild-type cells was not significantly changed by culture with insulin and/or glucose supplementation. These gene expressions were not significantly changed in transfectants. The expression of glucose transporter 2 mRNA was significantly increased in transfectants cultured for 72 h without insulin and/or glucose supplementation as compared with that of wild-type cells (Fig. 13A). Such an increase was not seen in transfectants cultured for 24 or 72 h with insulin and/or glucose supplementation.

DISCUSSION

Hyperlipidemia is induced in regucalcin transgenic rats [Yamaguchi et al., 2004], suggesting that lipid components are produced in the liver cells. This study was undertaken to determine whether overexpression of regucalcin stimulates the production of lipids in the cloned rat hepatoma H4-II-E cells in vitro. We found that overexpression of regucalcin could stimulate glucose utilization and lipid production in H4-II-E cells. This finding demonstrates that regucalcin can regulate glucose and lipid metabolism in liver cells.

Overexpression of regucalcin was found to stimulate the production of triglyceride and free fatty acid in the cloned rat hepatoma H4-II-E cells cultured without the supplementation of glucose in the absence of FBS, although it did not increase the consumption of glucose. This result suggests that regucalcin stimulates triglyceride and free fatty acid production in H4-II-E cells. When H4-II-E cells were cultured with the supplementation of glucose, overexpression of

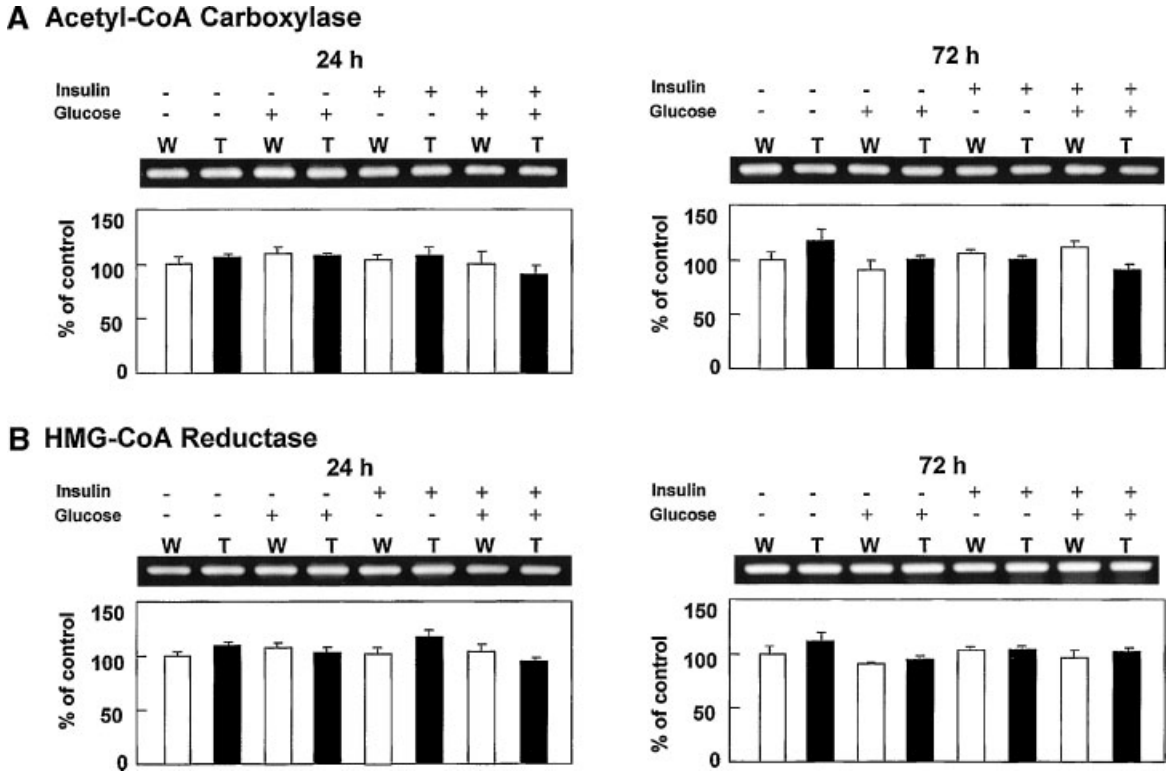

Fig. 11. Effect of insulin on acetyl-CoA carboxylase (**A**) and HMG-CoA reductase (**B**) mRNA expressions in the cloned rat hepatoma H4-II-E cells (wild-type; W) or regucalcin/pCXN2-transfected cells (T) cultured with or without glucose supplementation. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M) with or without supplementation of glucose (50 mg/ml of medium). The figure shows one of four experiments with separate samples. The densitometric data for mRNA levels were indicated as % of the control (none) of wild-type cells (mean ± SEM of four experiments). Data were not significant.

regucalcin caused a remarkable increase in glucose consumption and lipid production in H4-II-E cells. It is speculated that overexpression of regucalcin stimulates glucose utilization in H4-II-E cells, and that it promotes the production of triglyceride and free fatty acid which are linked to glucose metabolism in the cells in vitro.

Culture with insulin (10^{-7} M) caused a significant decrease in glucose consumption in wild-type cells, when H4-II-E cells were cultured in the presence of insulin without the supplementation of glucose. However, insulin had a stimulatory effect on glucose consumption in wild-type cells cultured with glucose supplementation. The effect of insulin was significantly suppressed in transfectants. Overexpression of regucalcin may have a suppressive effect on insulin-stimulated glucose consumption in H4-II-E cells.

Insulin had a stimulatory effect on the production of triglyceride in H4-II-E cells (wild-type) cultured with or without the supplementation of glucose. The stimulatory effect of insulin on triglyceride production in wild-type cells cultured with glucose supplementation was significantly suppressed in transfectants. Such an effect was not seen in transfectants cultured without glucose supplementation. Overexpression of regucalcin may have insulin resistance with higher glucose concentration in H4-II-E cells.

Insulin had a stimulatory effect on the production of free fatty acid in H4-II-E cells (wild-type) cultured with or without the supplementation of glucose. The stimulatory effect of insulin (10^{-7} M) on free fatty acid production was significantly suppressed in transfectants cultured with or without-glucose supplementation. These observations support the view that overexpression of regucalcin may have insulin resistance in H4-II-E cells.

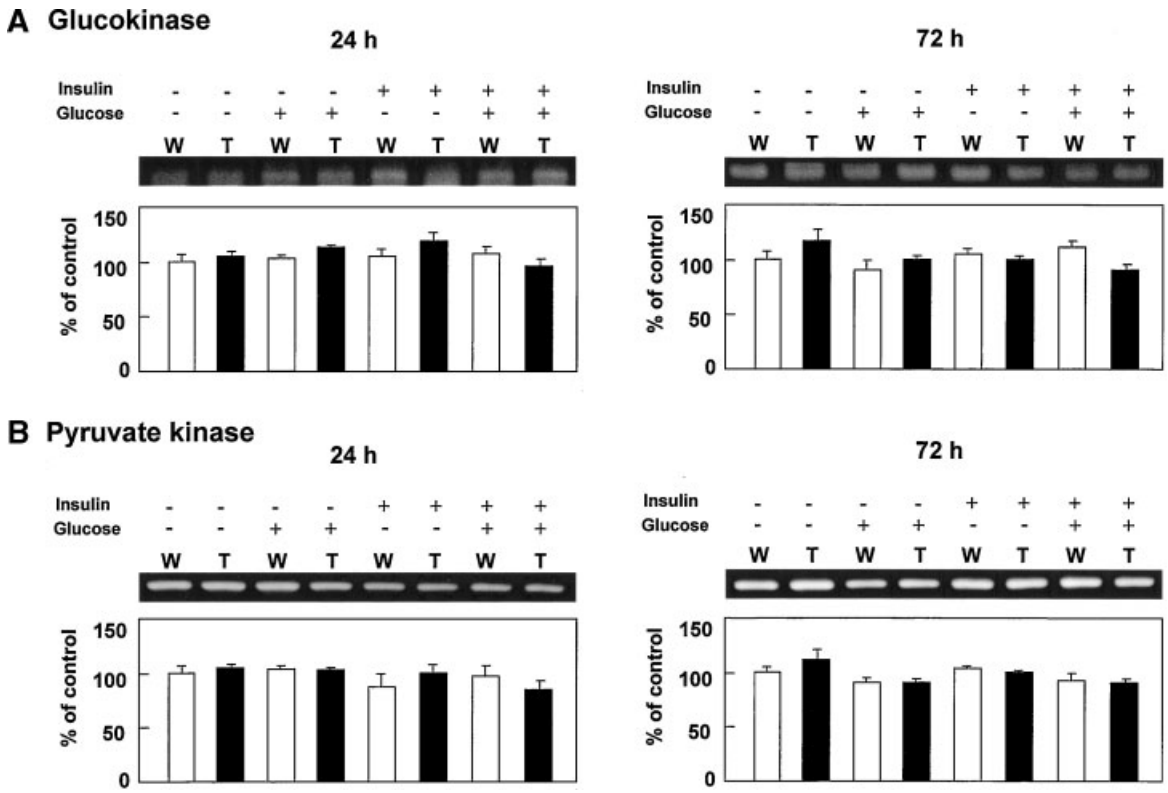

Fig. 12. Effect of insulin on glucokinase (**A**) and pyruvate kinase (**B**) mRNA expressions in the cloned rat hepatoma H4-II-E cells (wild-type; W) or regucalcin/pCXN2-transfected cells (T) cultured with or without glucose supplementation. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M) with or without supplementation of glucose (50 mg/ml of medium). The figure shows one of four experiments with separate samples. The densitometric data for mRNA levels were indicated as % of the control (none) of wild-type cells (mean ± SEM of four experiments). Data were not significant.

The gene expression of acetyl-CoA carboxylase, HGM-CoA reductase, glucokinase, and pyruvate kinase, which are rate-limiting enzymes related to glucose and lipid metabolism, in H4-II-E cells (wild-type) and transfectants was not significantly changed by culture with or without glucose supplementation in the presence of insulin. The expression of glucose transporter 2 mRNA was significantly increased in transfectants as compared with that of wild-type cells cultured without insulin and glucose supplementation. It is speculated that overexpression of regucalcin does not have a stimulatory effect on the gene expression of enzymes, which are related to glucose and lipid metabolisms, in H4-II-E cells. It is possible, however, that regucalcin has an activatory effect on various enzyme activities, which are related to glucose and lipid metabolism, in H4-II-E cells. In addition, it is speculated that regucalcin has a regulatory role in signal transduction of insulin in H4-II-E cells. Regucalcin has been shown to have suppressive effects on protein tyrosine kinase and insulin action in liver cells [Fukaya and Yamaguchi, 2005; Yamaguchi, 2005].

More recent studies show that insulin resistance may be modeled in H4-II-E liver cells in tissue culture with the use of the cytokine tumor necrosis factor-α and insulin [Solomon et al., 1997]. From the proteome of H4-II-E cells exposed to insulin and tumor necrosis factor-α, it is assumed that regucalcin is a role as proteins which are involved in insulin resistance [Solomon et al., 2005]. This is confirmed from our observations that overexpression of regucalcin has a role in insulin resistance in H4-II-E cells.

In conclusion, it has been demonstrated that overexpression of regucalcin enhanced glucose utilization and lipid production in the cloned rat

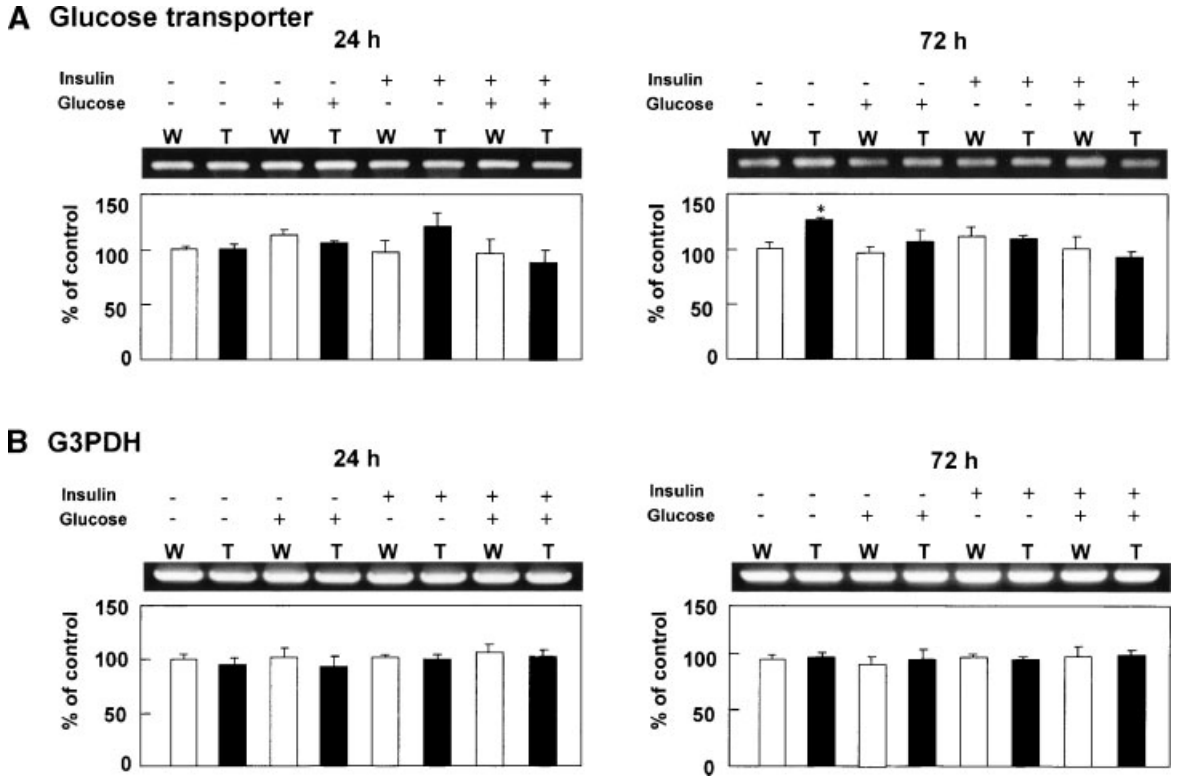

Fig. 13. Effect of insulin on glucose transporter (**A**) and G3PDH (**B**) mRNA expressions in the cloned rat hepatoma H4-II-E cells (wild-type; W) or regucalcin/pCXN2-transfected cells (T) cultured with or without glucose supplementation. Cells with subconfluency were cultured for 24 or 72 h in medium containing either vehicle or insulin (10^{-8} M) with or without supplementation of glucose (50 mg/ml of medium). The figure shows one of four experiments with separate samples. The densitometric data for mRNA levels were indicated as % of the control (none) of wild-type cells (mean ± SEM of four experiments). $^{*}P < 0.01$ compared with the control (none) value of wild-type cells.

hepatoma H4-II-E cells in vitro, and that it is involved in insulin resistance.

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