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Attenuation of Expression of γ-Glutamylcysteine Synthetase by Ribozyme Transfection Enhance Insulin Secretion by Pancreatic β Cell Line, MIN6

Hideaki Kondo,* Satoko Mori,† Hirofumi Takino,* Hiroshi Kijima,‡ Hironori Yamasaki,* Masako Ozaki,* Iida Tetsuya,† Yoshishige Urata,† Takahiro Abe,* Yasunori Sera,* Kenji Yamakawa,* Eiji Kawasaki,* Yoshihiko Yamaguchi,* Takahito Kondo,† and Katsumi Eguchi*

**First Department of Internal Medicine and †Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan; and ‡Department of Pathology, Tokai University School of Medicine, Kanagawa, Japan*

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Low levels of intracellular antioxidant enzyme activities as well as glutathione (GSH) concentrations have been described in pancreatic β cells. We examined the effects of intracellular GSH depletion on insulin secretion and the role of intracellular GSH in signal transduction in β cell line, MIN6 cells. Anti-γ-glutamylcysteine synthetase (γ-GCS) heavy subunit ribozyme was stably transfected to MIN6 cells to reduce intracellular GSH concentration. In the presence of 10 mM glucose, ribozyme-transfected cells (RTC) increased insulin secretion from 0.58 μg/10^6 cells/h in control cells (CC) to 1.48 μg/10^6 cells/h. This was associated with increased intracellular Ca^{2+} concentration in RTC, detected by fluo-3 staining. Our results demonstrated that intracellular GSH concentration might influence insulin secretion by MIN6 cells, and suggest that enhanced insulin secretion by β cells conditioned by chronic depletion of GSH is mediated by increased intracellular Ca^{2+} concentration.

Key Words: **insulin secretion; glutathione; redox; antioxidant enzymes; $[Ca^{2+}]_i$.**

Glutathione (γ-glutamylcysteinyl glycine, GSH), a polypeptide consisting of three amino acids, can metabolize free radicals acting as an intracellular antioxidant. Free radical hydrogen peroxide (H_2O_2) is catalyzed into H_2O and O_2 by supplementation of hydrogen from GSH. Therefore, GSH plays an important role in the regulation of intracellular reduction–oxidation (redox) state and protects cells against oxidative stress-induced cell damage (1, 2).

Intracellular redox state and GSH content influences several biological processes, such as signal transduction and enzyme activities (3). Protein-tyrosine-phosphatases (PTPs) contain a reactive cysteine residue in their active site (4). Thus, GSH can modify protein phosphorylation and affect the signal transduction (5). Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are important transcriptional factors, are also regulated through redox-induced modification of protein phosphorylation (6–9). Fos and Jun DNA binding is also regulated by redox state by means of a single conserved cysteine residue (Lys-Cys-Arg) in the DNA binding domains of the two proteins (6). These findings suggest that certain signal transduction pathways could be regulated by changes in intracellular redox, i.e., unbalanced oxidants and antioxidants.

The sensitivity of pancreatic β cells to oxidative stress induced by generation of free radicals or cytokine stimulation has been recently analyzed. Low levels of intracellular antioxidant-related enzyme activities as well as GSH content have been shown in islets or β cell lines compared to non-pancreatic tissues or non-β cell lines (10–12). However, the physiological significance of low level of redox state and antioxidant activities is not clear. Previous studies have also demonstrated that administration of thiol antioxidants, such as GSH, *N*-acetyl-L-cysteine, L-cysteine-methyl ester, and cystamine, enhanced glucose-induced insulin secretion from β cell lines and pancreatic islets (13–17). Although these studies provided evidence for the metabolic role of thiol antioxidants in the regulation of insulin secretion, these data were generated in experiments using exogenous thiols. The most of intracellular thiols is GSH. It is synthesized

Abbreviations used: GSH, glutathione (γ-glutamylcysteinyl glycine); γ-GCS, γ-glutamylcysteine synthetase; NF-κB, nuclear factor-κB; AP-1, activator protein-1; PKA, protein kinase A; PKC, protein kinase C.

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within the cells by 2 ATP-requiring steps catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase. The former enzyme, γ-GCS, which consists of two subunits, a catalytic heavy subunit and regulatory light subunit, catalyzes the rate limiting step of GSH synthesis (18, 19). For understanding the effect of intracellular GSH content on insulin secretion, direct evidence using gene knock out techniques are necessary. However, there has been no reports on the effect of change in GSH synthesis on insulin secretion.

To attenuate intracellular GSH concentration, we stably transfected anti-γ-GCS heavy subunit ribozyme into MIN6 cells, mouse pancreas β cell line, and characterized stable transfectants (20).

In the present study, using this cell line, we determined the direct effect of GSH depletion on insulin secretion and intracellular calcium concentration. Based on our findings, we discuss the important role of intracellular GSH in the regulation of insulin secretion by pancreatic β cells.

MATERIALS AND METHODS

Cell culture. Wild type MIN6, anti-γ-GCS heavy subunit ribozyme transfected MIN 6 and pHb plasmid only-transfected MIN 6 were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Rockville, MD) containing 25 mM glucose, 15% fetal calf serum (FCS), 3.4 mg/liter sodium bicarbonate, 75 μg/ml penicillin and 50 μg/ml streptomycin at 37°C in 5% CO_2 under 95% humidity. Wild-type MIN6 were generously provided by Prof. J. Miyazaki, University of Osaka. Anti-γ-GCS heavy subunit ribozyme transfected MIN 6 and pHb plasmid only-transfected MIN 6 were cultured with normal growth media containing 800 μg/ml geneticin (G418) (Sigma, St. Louis, MO) as previously described (20). We measured intracellular GSH concentration in all clones. Within the clones, which expressed ribozymes that were identified with RT-PCR, we selected two clones designated as RTC1 and RTC2 with the lowest GSH concentration, approximately by 50%, for subsequent experiments as previously described (20).

Northern blot analysis. A human γ-GCSh probe (764 base pairs) was prepared as described by Iida *et al.* (21). The probe was radiolabeled with [^{32}P]dCTP using Random Primer DNA labeling kit (Tanaka Co. Ltd., Tokyo, Japan). Isolation of cytoplasmic RNA and Northern blotting were essentially as described by Sambrook *et al.* (22). Cytoplasmic RNA isolated from cells were subjected to electrophoresis in 1% agarose gels containing 0.6 M formaldehyde, subsequently transferred to nylon membranes, and then hybridization with ^{32}P-labeled probes. Autoradiographed membranes were analyzed using a Fujix Bio-Analyser BAS-5000 (Fuji Film, Tokyo, Japan). After stripping, the membranes were rehybridized with ^{32}P-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe and the intensity of the bands was estimated. The relative radioactivity was expressed as a ratio of photostimulated luminescence (PSL) corrected by the intensity of GAPDH.

The concentration of GSH. The intracellular concentration of GSH was estimated by means of enzyme recycling as described by Beutler (23).

Insulin secretion. Insulin level was measured by ELISA method (Revis insulin, Shibaygi KK, Japan). RTC1 and CC were seeded at a density of 1×10^6/100-mm dish, and incubated with 15% FCS-containing DMEM supplemented by 10 or 25 mM glucose for 2 weeks. In order to prevent cell overconfluence, the cells were divided every week but maintained at the same glucose concentration. After 2-week culture, RTC1 and CC were re-seeded onto 30-mm dishes at 5×10^5 cells/well, and cultured in fresh 15% FCS-containing DMEM with glucose at the respective concentration for the next 24 h. Finally, 500 μl sample of the culture medium was frozen at −20°C until measurement of insulin level.

Intracellular Ca^{2+} expression. Each type of cells (1×10^5 cells) was cultured for 48 h with 15% FCS-containing DMEM with 10 or 25 mM glucose on 8-chamber slide glass (Tek, Nalge Nunc Inc., Naperville, IL), respectively. They were exposed to 5 mM acetoxymethyl ester fluo-3 (Molecular Probes, Eugene, OR) in anhydrous dimethyl sulfoxide (Wakojunyaku KK, Japan) for 1 h at 37°C and the long wavelengths were detected with an argon laser (514 nm) and fluo-3 optimal filters (LP520) in Laser scan microscopy (Carl Zeiss, Germany).

Intracellular ATP assay. Each type of cells (1×10^7 cells) was cultured for 48 h with 15% FCS containing DMEM with 25 mM glucose on 100-mm dishes. The media were removed and fresh normal media were added. After 10 min, cells were washed with ice-cold 0.9% NaCl and harvested with 0.25% trypsin. After centrifugation at 200*g* for 3 min, the pellet was washed with 0.9% NaCl. Then, we spectrophotometrically measured the cellular ATP concentration according to the method of Beutler (23).

Statistical analysis. The experimental data are expressed as means ± SD. Differences between groups were examined for statistical significance using the Student *t* test. Statistical differences were defined as $P < 0.05$.

RESULTS

We employed MIN-6 mouse islet cells without transfection of ribozyme (CC), transfected with anti-γ-GCS ribozyme clones 1 and 2 (RTC1 and RTC2), and transfected with disabled ribozyme (DRC). In order to reveal that anti-γ-GCS ribozyme could effectively function in RTC cells, the analysis of γ-GCS mRNA and intracellular GSH concentration was performed. The expression of γ-GCSh mRNA expressed as PSL% was 100 in CC, 105 in DRC, 60 in RTC1, and 48 in RTC2, shown in Fig. 1. The concentration of GSH was 3.57 ± 0.37 nmol/10^6 cells (mean ± SD of three independent analyses) in CC, 3.87 ± 0.37 nmol/10^6 cells in DRC, 1.98 ± 0.11 nmol/10^6 cells in RTC1, and 1.63 ± 0.25 nmol/10^6 cells in RTC2, respectively, shown in Fig. 2.

Insulin Secretion

To estimate the basal insulin secretion, CC and RTC were treated with 15% FCS-containing DMEM supplemented with 10 or 25 mM glucose for 2 weeks. The insulin secretion rate was defined as amount of insulin accumulating into the medium per one h. Treatment of CC with 25 mM glucose resulted in a decrease in insulin secretion by 63.7% relative to that observed in 10 mM glucose-treated CC in Fig. 3. Twenty five mM glucose-treated RTC secreted significantly less insulin, a reduction of 71.2%, compared to 10 mM glucose-treated RTC. This decrease was similar to that observed in CC. Interestingly, insulin secretion by RTC was significantly higher than that by CC in the presence of 10 and 25 mM glucose in Fig. 3.

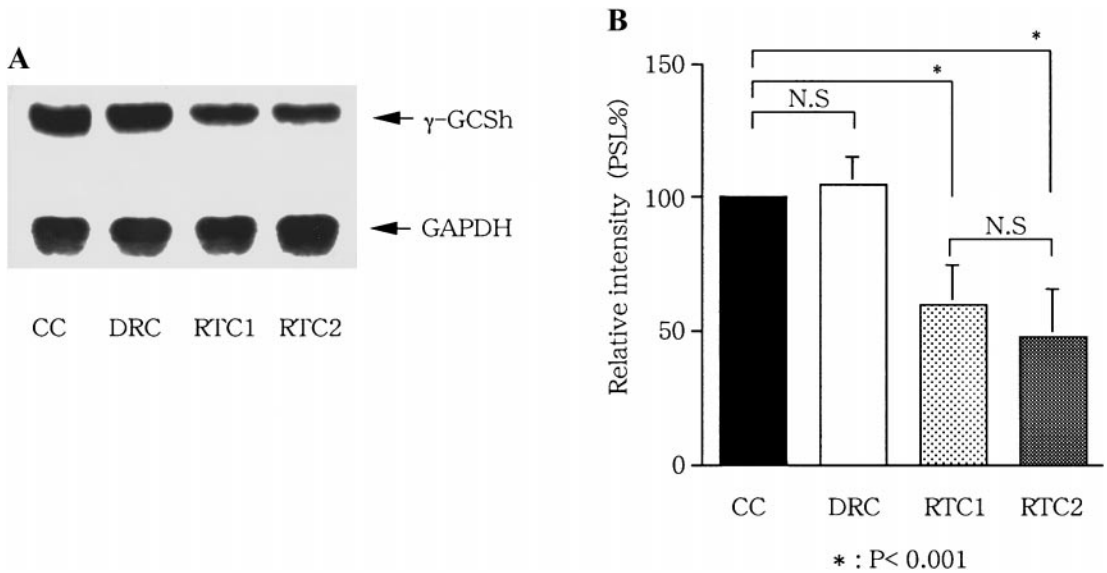

FIG. 1. Comparisons of mRNA levels of γ-GCSh and GAPDH genes in CC, DRC, RTC1, and RTC2 cells. (A) The bands corresponding to γ-GCS mRNA were indicated by an arrow in a representative result of Northern blot experiments. (B) The relative densities of the bands are expressed as PSL. Each plot is the mean ± SD from 3 independent experiments. *$P < 0.001$ versus CC cells.

Intracellular Ca^{2+} Expression

To assess the mechanism of enhanced insulin secretion by RTC1, the concentration of intracellular Ca^{2+} in each type of cells was visualized by acetoxymethyl ester fluo-3 as a ligand of intracellular Ca^{2+}. Although fluo-3 fluorescence showed a homogeneous distribution in CC and DRC throughout the cytoplasm of these cells, its intensity was weak as shown in Figs. 4A and 4B, respectively. In contrast, most RTC1 exhibited a strong fluorescence staining in the cytoplasm, with some cells even showing a condensed granular fluorescence staining in Fig. 4C.

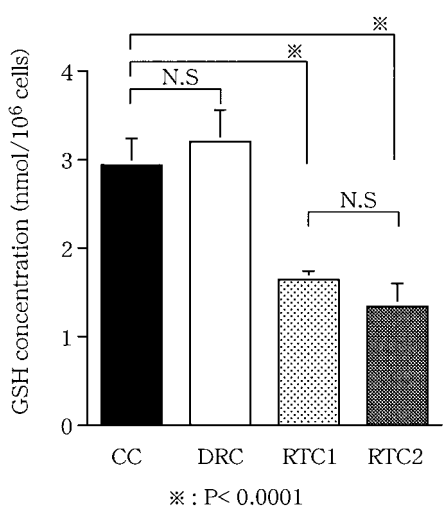

FIG. 2. Comparison of concentration of intracellular GSH in CC, DRC, RTC1, and RTC2 cells. Each value represents the mean of 3 independent experiments ± SD. ※, $P < 0.0001$ versus CC cells.

Intracellular ATP Assay

To assess the further mechanism of enhanced insulin secretion by RTC cells, the concentration of intracellular ATP was analyzed shown in Fig. 5. There was no significant difference in concentration of ATP between CC, DRC, RTC1, and RTC2 (135.2 ± 11.0, 139.7 ± 15.1, 132.6 ± 10.2, and 134.4 ± 9.5 nmol/mg protein, respectively).

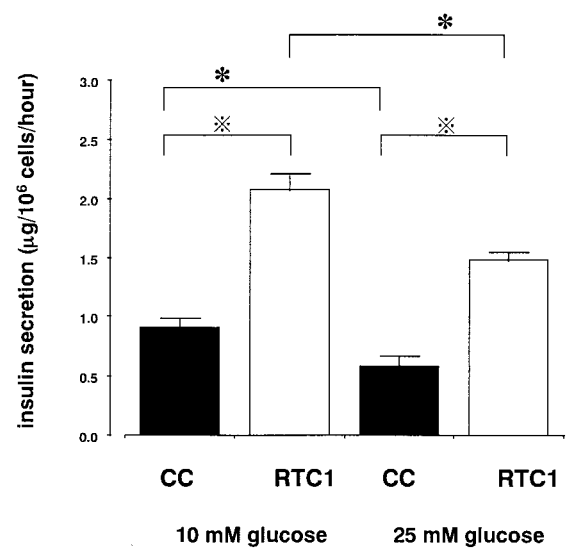

FIG. 3. Overall insulin secretion rate from CC cells (closed bars) and RTC1 cells (open bars) treated with 10 mM or 25 mM glucose concentrations. Each value represents the mean of 5 independent experiments ± SD. *$P < 0.0001$ for CC vs RTC. ※, $P < 0.001$ for 10 mM vs 25 mM glucose concentration.

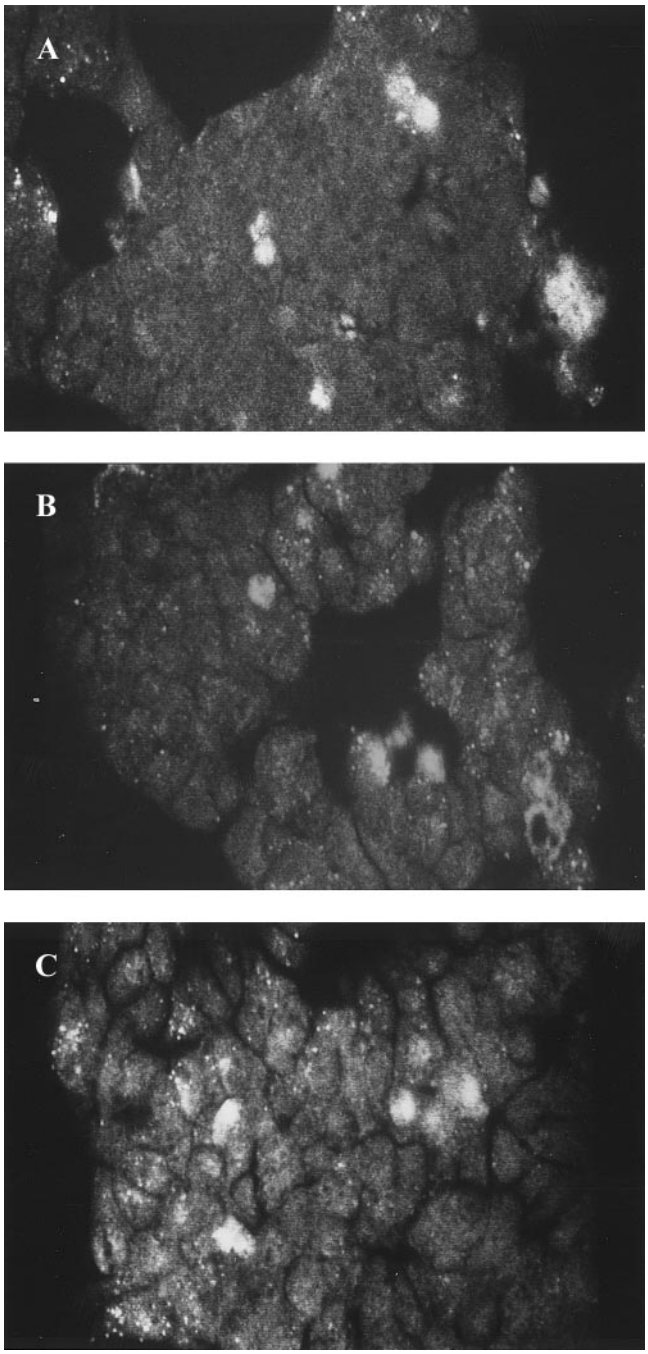

FIG. 4. Intracellular Ca^{2+} concentration determined by using acetoxymethyl ester fluo-3 in CC (A), DRC (B), and RTC1 (C) cells with a magnification ×400.

DISCUSSION

The present study showed that low intracellular GSH levels in β cell line were associated with enhanced insulin secretion and that such effect was mediated, at least in part, by increased intracellular Ca^{2+} concentration.

Previous studies have demonstrated that administration of thiols, such as GSH, *N*-acetyl-L-cysteine, L-cysteine-methyl ester, and cystamine, augmented the insulin secretion by β cell lines and pancreatic islets (14, 15, 17). Thiols also influence the secretion of other hormones by endocrine cells or primary cultured cells such as growth hormone, prolactin and parathyroid hormone (24–26). These results indicate that GSH and/or cysteine derivatives may act as a stimulator for endocrine cells on hormone secretion. However, our studies were the results that were assessed on the relationship between the decreased GSH condition and insulin secretion in β cells. Theirs and ours are basically different. They have reported the effects of thiols administration exogenously and we have reported here how the chronically decreased GSH affected insulin secretion.

To identify the effect of intracellular GSH on insulin secretion, a direct evidence using gene knockout technique is required. RTC which express anti-γ-GCS ribozyme gene show reduced expression of γ-GCS mRNA and low intracellular GSH concentration (20). Utilizing these stable transfected cells, we demonstrated here that decreased GSH levels resulted in increase secretion of insulin.

The intracellular concentration of GSH and its related enzyme activity are decreased in β cell lines and pancreatic islets, compared with other tissues or organ-derived cell lines (10–12). Although the exact mechanisms of decreased GSH and its related enzyme activities in insulin-secreting cells are not understood at present, GSH might act as an inhibitory agent to lower insulin secretion.

Recent reports have suggested that the redox regulation by GSH and/or cysteine derivatives is associated with alteration of gene transcription and enzyme activities. These SH-group-containing molecules regulate the transcriptional activities by reducing the disulfide

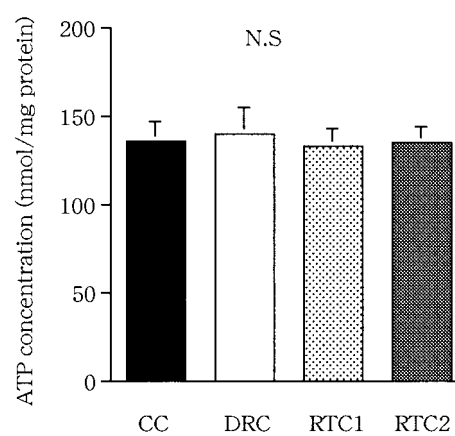

FIG. 5. Intracellular ATP concentrations in CC, DRC, RTC1, and RTC2 cells. Results were expressed as means ± SD from 3 independent experiments.

bond(s) present in the protein configuration of the transcriptional factors. This is similar for activities of some enzymes. Such enzymes, e.g., protein tyrosine phosphatases, are known to contain a cysteine residue in the catalytic domain. The protein structure can potentially be by the reductive ability of intracellular redox state, e.g., GSH (6–9).

Our results also showed increased basal level of intracellular Ca^{2+} concentration in RTC with low GSH content and the mechanism of increased intracellular Ca^{2+} was independent of intracellular ATP concentration. In pancreatic β cells, at least three possible pathways lead to insulin secretion after glucose stimulation: (1) K_{ATP} channel-and Ca^{2+}-dependent pathway, (2) K_{ATP} channel-independent and Ca^{2+}-dependent pathway (27, 28), and (3) Ca^{2+}-independent pathway (29). Persistent glucose stimulation (25 mM glucose) of MIN6 cells in the present study would evoke an increase in the cytoplasmic Ca^{2+} probably through K_{ATP} channel-independent pathway (27, 28). This pathway has been shown to involve PKA and PKC. In this regard, activation of PKA is thought to elevate intracellular Ca^{2+} concentration independent of K_{ATP} channel, and activate PKC and increase insulin secretion. PKA related pathway is also implicated in Ca^{2+} independent pathway (29). A possible explanation for the increased Ca^{2+} concentration in RTC would be that the induction of PKA activation might be induced by intracellular redox regulation, i.e., reduced GSH content. In addition, it is also possible that GSH depletion might also activate insulin gene expression such as in RTC. Interestingly, cAMP responsive element binding protein (CREBP) binding to the insulin promoter region has been shown to be affected by PKA activation (30, 31).

Although the biochemical relevance of intracellular GSH concentration to insulin secretion by β cells remains to be determined, the present data suggest that a balance between intracellular reduction and oxidation in β cells might be an important physiological factor in the regulation of insulin secretion.

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