Involvement of thioredoxin in the regulation of growth hormone secretion in rat pituitary cell cultures

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Hata, Ikue, Yosuke Shigematsu, Yusei Ohshima, Hirokazu Tsukahara, Kazuo Fujisawa, Masahiro Hiraoka, Hajime Nakamura, Hiroshi Masutani, Junji Yoido, Fumikazu Kotsuji, Masakatsu Sudo, and Mitsufumi Mayumi. Involvement of thioredoxin in the regulation of growth hormone secretion in rat pituitary cell cultures. Am J Physiol Endocrinol Metab 281: E269–E274, 2001.—We report here an examination of the effect of thioredoxin (TRX) on the secretion of growth hormone (GH) from rat anterior pituitary cells in vitro. Treatment of rat pituitary cells with growth hormone-releasing factor (GRF), but not GH, led to a significant increase in intracellular TRX protein levels. GRF, recombinant human TRX (rhTRX), and a combination thereof were all shown to induce immediate GH secretion from pituitary cells, as evidenced by perfusional experiments. RhTRX, but not other reducing agents such as β-mercaptoethanol and N-acetyl-L-cysteine, augmented GRF-stimulated and -unstimulated GH secretion from rat pituitary cells in a dose-dependent manner. RhTRX did not significantly affect the GH mRNA expression of pituitary cells stimulated in the presence or absence of GRF. In addition, rhTRX-augmented GH secretion was not significantly affected by the presence of cycloheximide. Collectively, these findings suggest that TRX is induced by stimulation with GRF and plays a regulatory role in GH secretion from rat anterior pituitary cells by enhancing the secretion of stored GH, rather than by the synthesis of GH.

IT HAS RECENTLY BEEN SHOWN that reduction/oxidation (redox) reactions are intimately involved in the control of biological processes including the functional modulation of transcription factors (25, 32). In the case of the endocrine system, the cellular redox state appears to regulate the secretion and action of hormones. With regard to the hypothalamic-pituitary axis, a critical role for nitric oxide (NO), an endogenous redox modulator (34), in the regulation of growth hormone (GH) secretion has been proposed. We and others (7, 13) have recently reported that cultured rat pituitary cells tonically produce NO, which, in turn, blunts the growth hormone-releasing factor (GRF)-induced GH secretion through a guanosine 3′,5′-cyclic monophosphate (cGMP)-independent mechanism.

An important constituent of the oxidant buffering system that controls the cellular redox state is thioredoxin (TRX), a 12-kDa protein with a redox-active disulfide/dithiol in the conserved active site sequence Cys-Gly-Pro-Cys (9, 25). This molecule has a variety of activities including serving as a hydrogen donor for various intracellular molecules (15, 24). Evidence has accumulated that suggests the presence of a control mechanism by the TRX system in certain endocrine systems (3, 8). For example, in the hypothalamic-pituitary-adrenal axis, TRX modulates cellular glucocorticoid responsiveness (6, 22). In the human ovary, adult T-cell leukemia-derived factor, the human form of TRX, exists and may participate in steroid hormone production (11). Recent immunohistological studies (28, 29) have demonstrated an intense level of staining for TRX in the pig anterior pituitary gland, supporting the contention that the TRX system may play a role in the regulation of GH secretion. However, to date, no data are available regarding the role of TRX in the GRF-GH axis.

In the present study, we report on an investigation of the effect of GRF on the synthesis of TRX in rat anterior pituitary cells and the regulatory role of TRX in GH secretion from these cells. Our findings present the first evidence that suggests that the TRX system, which is stimulated by GRF, acts as an enhancer of GH secretion in the rat anterior pituitary gland.

MATERIALS AND METHODS

Pituitary cell dispersion. Anterior pituitary glands were collected from male Sprague-Dawley rats, aged 6–8 wk (Clea Japan, Tokyo, Japan). For ≥3 days before decapitation, the animals were kept in our animal facilities at 24°C on a 12:12-h light-dark cycle and received food and water ad libitum. Primary pituitary cell cultures were prepared as described previously (12, 14), with some modifications. Briefly, rat anterior pituitaries were finely minced and incubated with 0.3% type I collagenase (Sigma Chemical, St.

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Louis, MO) and 0.0009% DNase (Sigma Chemical) in Hanks’-HEPES buffer containing 0.4% BSA at 37°C for 20–30 min. The pituitary suspension was triturated through Pasteur pipettes at 5-min intervals during the incubation.

The dispersed cells were washed three times with DMEM (GIBCO-BRL, Life Technologies, Rockville, MD) containing 10% fetal bovine serum (referred to hereinafter as the culture medium), resuspended in the culture medium, and used for the primary culture. The yield of cells was 7–10 × 10⁶ cells/pituitary, and the viability was ~90%, based on the trypan blue exclusion test. The experimental protocol was approved by the animal care committee of our university.

**Western blot analysis of GRF- or GH-induced TRX production.** Dispersed pituitary cells were cultured at 1.0–1.5 × 10⁶ cells·ml⁻¹·well⁻¹ in 24-well culture plates (Corning, New York, NY) for 4–5 days. The confluent grown cells were then reincubated with 1 ml of the fresh culture medium/well for 24 h in the presence of 10⁻² M human GRF-(1–44) (GRF; Peptide Institute, Osaka, Japan) or 500 ng/ml rat GH (kindly provided by Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) during the last 0, 6, 12, and 24 h of the culture periods. The TRX contents in the cells were then determined by Western blot analysis as described previously (31). Briefly, the cultured cells were washed three times with ice-cold PBS and then treated with a solubilizing buffer (0.5% octylphenoxyl poly-ethoxylethanol (Nonidet P-40), 10 mM Tris·HCl, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.111 U/ml aprotinin, and 0.02% NaN₃) on ice for 30 min. The resultant lysates were centrifuged at 10,000 g for 10 min, and the supernatants were used for SDS-PAGE. The concentrations of protein in the supernatants were determined by the modified Lowry method (Bio-Rad Laboratories, Hercules, CA). Equal amounts of proteins (8 or 10 μg) were applied to each lane. After electrophoresis, proteins were electrically transferred onto a nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with 10% skim milk and 2% BSA and then incubated with rabbit antiserum to murine TRX (1:2,000 dilution) at 4°C overnight, followed by horseradish peroxidase-linked goat anti-rabbit immunoglobulins (1:100 dilution, according to the manufacturer’s instructions) (ENVISION+, Dako Japan, Kyoto, Japan). Detection of the antigen-antibody complex was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer’s instructions. Quantification of TRX was performed by densitometric analysis with an imaging densitometer (NIH image).

**Effect of TRX, other reducing agents, and cycloheximide on GH secretion.** Perfusion experiments were performed as previously described (14), with minor modifications. Briefly, dispersed pituitary cells were cultured with preswollen Cytodex microcarriers type 3 (Amersham Pharmacia Biotech) in the culture medium at a ratio of 1.0 × 10⁶ cells to 10 mg microcarriers in 3-cm siliconized glass dishes. After 4–5 days of culture, the cells were packed into Lucite columns containing 5 mg microcarriers and placed in a 37°C incubator. Three columns were perfused simultaneously with DMEM at 0.5 ml/min and stimulated with DMEM containing GRF (10⁻⁹ M) or rhTRX (100 μg/ml; Ajinomoto, Kawasaki, Japan) or both for a 5-min period at 90-min intervals. The column effluents were collected every 2 min by means of a fraction collector and stored at −80°C for measurement of GH concentrations.

Dispersed pituitary cells in the culture medium were also seeded at a concentration of 6.0 × 10⁴ cells·500 μl⁻¹·well⁻¹ in 48-well culture plates (Becton-Dickinson, Franklin Lakes, NJ). After 4–5 days of culture, the confluent grown cells were rinsed twice with DMEM and incubated for another hour with 300 μl of DMEM containing various concentrations of rhTRX (0–100 μg/ml) and/or GRF (10⁻⁹ M). The effect of β-mercaptoethanol and N-acetyl cysteine at the concentrations of 1 and 10 μM rhTRX in some experiments; 1 and 10 μM rhTRX are equivalent to 12 and 120 μg/ml. In certain experiments, pituitary cells were incubated with rhTRX and/or GRF in the presence of cycloheximide (200 μM; Sigma Chemical) to prevent de novo protein synthesis. The medium was then collected and stored at −80°C until assayed for GH concentrations.

**Measurement of GH concentrations.** The concentrations of GH of the medium were measured by RIA, which was done in duplicate with a kit that was kindly provided by Dr. A. F. Parlow. RhTRX and other reducing agents at the concentrations used had no effect on the measurement of GH (data not shown).

**Northern blot analysis of GH mRNA.** Dispersed pituitary cells were cultured at a density of 2.5–4.1 × 10⁶ cells/well in 2 ml of the culture medium in 6-well culture plates (Becton-Dickinson). After 4–5 days, the confluent grown cells were rinsed twice with DMEM and incubated with 2 ml of DMEM containing rhTRX (100 μg/ml) and/or GRF (10⁻⁹ M) for additional 4 h. At the end of the incubation, cells were washed twice with ice-cold PBS. Total cellular RNA was extracted with RNA isolation reagent (Isogen, Nippon Gene, Tokyo, Japan). Three micrograms of RNA were separated on a 1% agarose gel in 0.2 M 3-(N-morpholino)propanesulfonic acid buffer and then transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech). Hybridizations were performed using the rat GH cDNA probe labeled with deoxy(α-th²P)cytidine 5’-triphosphate, which was kindly provided by the Bioscience Research Institute of JCR Pharmaceuticals (Kobe, Japan). A final series of washes was carried out at 2× saline-sodium citrate (SSC) buffer/0.1% SDS at room temperature, and 0.1 × SSC/0.1% SDS at 47°C. Quantification of GH mRNA was performed by densitometric analysis with the use of the NIH image.

**Statistical analysis.** All data are presented as means ± SE. Statistical comparisons were performed by one-way analysis of variance (ANOVA), with the use of the Bonferroni-Dunn test, and by the Student’s t-test for the effect of cycloheximide and the effect of rhTRX on GH mRNA. P values <0.05 were considered statistically significant.

**RESULTS**

**Effect of GRF on intracellular TRX concentration.** The effect of GRF on intracellular TRX levels was first examined in rat pituitary cells. Because our preliminary experiments showed that the incubation of pituitary cells with GRF for 6 h was insufficient to induce significant increases in TRX levels (data not shown), we determined TRX levels after 12 and 24 h of incubation with GRF in the following experiments. As shown in Fig. 1, A and B, the cultured rat pituitary cells contained detectable amounts of TRX, and stimulation of the cells with GRF significantly increased the intracellular TRX protein levels. The mean TRX protein levels of cells stimulated with GRF during the last 12 and 24 h of the culture period were 2.6 and 2.3 times higher, respectively, than that observed in the controls cultured without GRF throughout the culture period (Fig. 1B). These results indicate that GRF caused an increase in TRX protein levels within 12 h. In contrast, the TRX protein levels were not altered by GH stimu-
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Fig. 1. Effects of growth hormone-releasing factor (GRF) and growth hormone (GH) on intracellular thioredoxin (TRX) protein levels. Confluently grown rat anterior pituitary cells were incubated for an additional 24 h with 10^{-7} M GRF (A and B) or 500 ng/ml of GH (C and D) during the indicated time periods of the incubation. Zero indicates that the cells were incubated for 24 h in the absence of GRF or GH as a control. A and C: representative Western blot analysis, the results of which were confirmed by repeated experiments. B and D: intracellular TRX protein levels (means ± SE of 3 independent experiments) of cells stimulated with GRF (B) or GH (D) for the indicated hours. Data are expressed as percentages of the intensity of the control bands (time 0). *P < 0.05 vs. time 0.

Fig. 2. GH secretion from cultured rat anterior pituitary cells in response to short-term treatment. Cultured cells, packed into columns, were perifused with the medium and exposed to GRF (10^{-9} M; ○), recombinant human (rh)TRX (100 μg/ml; ▲), or both (●) for 5 min. The concentrations of GH in the effluents, collected at 2-min intervals, were measured. Changes in GH secretion (means ± SE of quadruplicate determinants) are expressed as percentages of the basal GH concentration, which is the mean of 5 fractions just before stimulation. Results were confirmed by 3 independent experiments, and representative data are shown. There is no statistically significant difference among the peaks of GH secretion induced by the 3 different stimuli.

Fig. 3. TRX-induced GH secretion from cultured rat anterior pituitary cells. Confluently grown pituitary cells were incubated in culture wells for an additional 1 h with the indicated concentrations of rhTRX in the presence (●) or absence (○) of 10^{-9} M of GRF. Data in A show the concentrations of GH (means ± SE of quadruplicate determinants) of the representative experiment. Increases in GH concentration (means ± SE of 5 independent experiments) stimulated with rhTRX in the presence and absence of GRF are shown in B and C, respectively, as the percentages of those cultured without rhTRX. *P < 0.05; **P < 0.01 vs. without TRX.

Effect of TRX on GH secretion. The possibility that TRX influenced GH secretion from rat pituitary cells was then examined. In perifusion experiments, the GH secretion significantly increased immediately after stimulation at any time examined (Fig. 1, C and D). These results suggest that the GRF-induced increase in TRX is not due to an indirect effect via GH.
stimulation with $10^{-9}$ M GRF, 100 $\mu$g/ml rhTRX, or both (Fig. 2). The GH secretion reached maximum within 5 min after the start of stimulation with each stimulus and decreased rapidly after the cessation of the 5-min stimulation. The maximum GH secretion generated by GRF plus rhTRX was higher than those by GRF or rhTRX alone but was not statistically significant. The stimulation was repeated 3 or 4 times after 90-min intervals in each experiment, and the results were nearly the same (data not shown). The cell viability at the end of the perfusion experiments was 90–93%, regardless of the stimuli.

The dose response of rhTRX on GH secretion was then examined. Rat pituitary cells were stimulated for 1 h with different concentrations of rhTRX in the presence or absence of 200 $\mu$M cycloheximide, as indicated. Changes in GH concentration (means ± SE of 4 independent experiments) are shown as percentages of those cultured without rhTRX and cycloheximide. n.s., Non-significant.

Effect of cycloheximide on GH secretion. Because the TRX-induced augmentation of GH secretion was observed immediately after stimulation with rhTRX (Figs. 2 and 3), it is likely that TRX shows its effect through the secretion of intracellularly stored GH but through an augmentation in the de novo synthesis of GH. To confirm this possibility, pituitary cells were stimulated with rhTRX in the presence of cycloheximide. As shown in Fig. 4, cycloheximide had no significant effect on the amounts of GH secreted by the cells treated with rhTRX and/or GRF.

Effect of TRX on GH mRNA expression. As shown in Fig. 5, rhTRX had no effect on GH mRNA levels in rat pituitary cells.

Fig. 4. Effect of cycloheximide on GH secretion. Confluently grown pituitary cells were treated with 100 $\mu$g/ml rhTRX (A), or with 100 $\mu$g/ml rhTRX plus $10^{-9}$ M GRF (B) for 1 h in the presence or absence of 200 $\mu$M cycloheximide, as indicated. Changes in GH concentration (means ± SE of 4 independent experiments) are shown as percentages of those cultured without rhTRX and cycloheximide. n.s., Non-significant.

Fig. 5. Effect of GRF and TRX on GH mRNA expression. Expression of GH mRNA of rat pituitary cells stimulated with rhTRX (100 $\mu$g/ml), GRF ($10^{-9}$ M), or both for 4 h was determined by Northern blot analysis and densitometric analysis. The contents of GH mRNA were corrected according to those of $\beta$-actin. A: representative autoradiograph; data were confirmed by repeated experiments. B: changes in GH mRNA levels (means ± SE of 3 independent experiments) are expressed as the percentages of those cultured without rhTRX or GRF.

Fig. 6. Effects of reducing agents on GH secretion. Confluently grown rat pituitary cells were incubated for 1 h with or without $\beta$-mercaptoethanol (○), N-acetyl-L-cysteine (●) and rhTRX (▲) at concentrations of 1 and 10 $\mu$M in the presence (A) or absence (B) of GRF ($10^{-9}$ M). Changes in GH concentrations of medium are shown as percentages of those cultured without the reducing agents. Shown are means ± SE of 4 independent experiments, each of which is the mean of quadruplicated determinants. **$P < 0.01$ vs. without reducing agents.
pituitary cells cultured with or without GRF, even after 4 h. The data also suggest that rhTRX enhances the secretion of stored GH but not the de novo synthesis of GH.

Effects of other reducing agents on GH secretion. Although rhTRX significantly augmented GH secretion, β-mercaptoethanol and N-acetyl-L-cysteine at concentrations of 1 and 10 μM had no significant effect on GH secretion (Fig. 6). The viability of cells was not changed regardless of the treatments (data not shown).

DISCUSSION

The physiological secretion of GH is generally thought to be regulated primarily by the two hypothalamic peptides GRF and somatostatin (5, 35), and redox modulation of disulfide bonds of hormones and receptors has been shown to influence GH secretion (4, 21, 36) as well as hormone-induced receptor activation (6, 8) and signal transduction (23). A redox-modulatory substance, NO, modulates the GRF-stimulated secretion of GH from rat pituitary cell cultures (13). TRX, another important redox-modulatory substance, has recently been identified in pig anterior pituitary gland by immunohistochemical analysis (28, 29). Immunoblotting analysis suggests that this is the case in calf and rat pituitary as well. This suggests that the TRX system is widely involved in the regulation of GH secretion from anterior pituitary gland. The data reported herein represent the first implication of the GRF-TRX-GH axis in rat pituitary gland.

The present findings show that stimulation with GRF increased TRX levels in rat anterior pituitary cells, and extrinsic rhTRX induced secretion of GH from there. Although the issue of whether GRF directly induces TRX production or indirectly induces it through induction/augmentation of other proteins is not clear, our results show that GH is not possibly the indirect inducer. The fact that TRX can be induced through a cAMP-dependent pathway (37), and that GRF is capable of activating a cAMP-dependent pathway in pituitary cells (1, 2, 4), suggests that GRF directly induces the synthesis of TRX protein in rat pituitary cells through a cAMP-dependent pathway.

Several possible explanations exist for the rhTRX-induced augmentation of GH secretion from rat pituitary cells, including the induction of de novo GH synthesis, augmentation of secretion of stored GH, or both. The fact that stimulation with rhTRX immediately elicited GH secretion (Fig. 2), that cycloheximide did not influence rhTRX-induced GH secretion (Fig. 4), and that rhTRX did not appear to upregulate GH mRNA expression (Fig. 5), suggests that rhTRX augments GH secretion by increasing the secretion of stored GH but not through the induction of the de novo synthesis of GH. GH is stored in pituitary secretory granules in high concentrations in the form of intermolecular disulfide-bonded oligomers (20), and the release of GH and prolactin from isolated pituitary secretory granules is increased by the presence of glutathione and other thiol-reducing agents, probably through the disruption of disulfide bonds in the hormone oligomers and/or granule membrane proteins (18, 19). It is likely that TRX enters cells (33) and that its role in GH secretion involves its strong reducing activity (10). However, because two other reducing agents, β-mercaptoethanol and N-acetyl-L-cysteine, failed to enhance GH secretion in our study, it is possible that TRX may exert its role through mechanisms other than simple reduction as well.

On the other hand, Lefrançois et al. (16) showed that reducing agents such as glutathione and dithiothreitol suppressed the coupling of GRF receptor with GRF. Other studies have also shown that the coupling of secretin or glucagon receptors with their ligands were decreased by thiol-reducing agents (17, 27, 30). Although such an inhibitory effect of TRX was not apparent in our experiments, it is possible that TRX, as well as other thiol-reducing physiological substances, has two opposing effects and regulates the GH secretion in a complex manner. Further analyses of the redox regulation of the GRF-GH axis may provide a better understanding of the characteristic pulsatile secretion of GH.

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