Long Exposure to High Glucose Concentration Impairs the Responsive Expression of \( \gamma \)-Glutamylcysteine Synthetase by Interleukin-1\( \beta \) and Tumor Necrosis Factor-\( \alpha \) in Mouse Endothelial Cells*

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Yoshishige Urata\( \dagger \), Hidefumi Yamamoto\( \ddagger \), Shinnji Goto\( \ddagger \), Hideki Tsushima\( \ddagger \), Shouichi Akazawa\( \ddagger \), and Takahito Kondoh\( \ddagger \)

From the \( \dagger \)Department of Pathological Biochemistry, Atomic Disease Institute, and the \( \ddagger \)First Department of Medicine, Nagasaki University School of Medicine, Nagasaki 852, Japan.

To elucidate the pathological metabolism of glutathione synthesis in diabetic endothelial cells, we studied the expression of \( \gamma \)-glutamylcysteine synthetase (\( \gamma \)-GCS) using a mouse vascular endothelial cell line.

Exposing normoglycemic endothelial cells to tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) or interleukin-1\( \beta \) (IL-1\( \beta \)) increased the activity and the mRNA expression of \( \gamma \)-GCS. The addition of inhibitors for nuclear factor \( \kappa \)B (NF-\( \kappa \)B) to the cells caused a loss of the \( \gamma \)-GCS mRNA expression in response to TNF-\( \alpha \).

A shift of the concentration of glucose in the medium from 5.5 to 28 mM glucose and a following incubation for 7 days decreased the expression of \( \gamma \)-GCS mRNA. These cells showed no apparent responses of \( \gamma \)-GCS mRNA or the activity of NF-\( \kappa \)B to TNF-\( \alpha \) or IL-\( \beta \). Increase in the GSH concentration of the cells treated with 28 mM glucose restored the expression of \( \gamma \)-GCS mRNA and its response to TNF-\( \alpha \) or IL-\( \beta \), suggesting that redox regulation is involved in the expression of \( \gamma \)-GCS.

In summary, the expression of \( \gamma \)-GCS is regulated by TNF-\( \alpha \) or IL-1\( \beta \) in endothelial cells mediated by NF-\( \kappa \)B stimulation, and impairment of the regulation of \( \gamma \)-GCS in hyperglycemic cells may be a cause of medical complications that develop in diabetes mellitus.

Endothelial cells play important roles in selective transport, anticoagulation, lipid metabolism, vascular tension, vascularization, and immunological regulation. The secretion of some cytokines or growth factors from endothelial cells and their binding to specific receptors mediate these functions (1). In diabetes mellitus, endothelial cell damage is believed to be a significant contributing factor in the development of medical complications. Oxidative stress is a factor involved in cellular injury (2). Increases in the production of oxygen radical species or decreases in the scavenging activity against oxidative stress may play crucial roles in the development of pathological conditions (3).

Glutathione (\( \gamma \)-glutamylcysteinyI glycine, GSH) participates in many biological processes such as the metabolism of amino acids containing sulfur, biosynthesis of leukotrienes and DNA, and in the cellular defense system against oxidative stress by reducing the disulfide linkage of proteins and other cellular molecules or by scavenging free radicals and reactive oxygen intermediates (4). The physiological role of GSH as an antioxidant has been described and substantiated in numerous disorders reflecting increased oxidation as a result of an abnormal redox metabolism. GSH is synthesized in most mammalian cells by the activity of two ATP-requiring GSH-synthesizing enzymes, \( \gamma \)-glutamylcysteine synthetase (\( \gamma \)-GCS) and glutathione synthetase, \( \gamma \)-GCS catalyzes the rate-limiting step of GSH synthesis (5). This enzyme has been purified and the structure of the DNA from the rat kidney has been determined (6). \( \gamma \)-GCS synthesis is also thought to be an important factor in cellular defense against stress such as radiation and drug resistance (7). We showed evidence that the concentration of GSH and the activity of \( \gamma \)-GCS are decreased in endothelial cells from experimental diabetic rabbits (8) and that the expression of \( \gamma \)-GCS mRNA is sensitive to stress such as heat shock or chemical insults (9). However, the pathological significance of the expression of \( \gamma \)-GCS in the development of diabetic complications in endothelial cells is not known.

Tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and interleukin-1\( \beta \) (IL-1\( \beta \)) are cytokines initially produced by monocyte-macrophages in response to endotoxin and various mitogens (10). These cytokines mediate multiple immunological and inflammatory events, such as increases in endothelial cell surface procoagulant and plasminogen activator inhibitor, or vasodilatory and angiogenic effects (11). The effect of IL-1\( \beta \) is potentiated by TNF-\( \alpha \) (12), which activates nuclear factor \( \kappa \)B (NF-\( \kappa \)B) formation (13). NF-\( \kappa \)B is involved in the expression of dimeric transcriptional factors (14) and through its dissociation from its inhibitor, I\( \kappa \)B (13), transcriptionally activates various cellular genes involved in immune response, inflammation, oxidative stress, and embryonic development (15–17). NF-\( \kappa \)B is characterized as a heterodimer with two subunits, p50 and p65 (18).

To understand the regulatory mechanisms for GSH synthesis under diabetic conditions, we studied changes in the responsiveness of \( \gamma \)-GCS to IL-1\( \beta \) and TNF-\( \alpha \) in mouse endothelial cells cultured with a high concentration of glucose. We also investigated the activation of NF-\( \kappa \)B and \( \gamma \)-GCS expression by TNF-\( \alpha \) are linked.

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\( \dagger \)To whom all correspondence should be addressed: Professor and Director, Dept. of Pathological Biochemistry, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki 852, Japan. Tel.: 81-958-49-7100; Fax: 81-958-49-7097.

\( \ddagger \)The abbreviations used are: \( \gamma \)-GCS, \( \gamma \)-glutamylcysteine synthetase; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \); IL-1\( \beta \), interleukin-1\( \beta \); 8-OHdG, 8-hydroxydeoxyguanosine; MHE, mouse hemangioendothelioma; SOD, superoxide dismutase; PSL, photostimulated luminescence; TLCK, 7-amino-3-chloro-3-tosylamide-2-hepatanone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; NF-\( \kappa \)B, nuclear factor \( \kappa \)B.
Materials—Glutathione reductase was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Dulbecco's modified Eagle's medium, fetal calf serum and NF-κB binding protein detection system were from Life Technologies, Inc. \(^{32}P\)ATP was from DuPont NEN. Recombinant human IL-1β was from Otsuka Pharmaceutical Co. (Tokushima, Japan), and TNF-α was from Dainippon Pharmaceutical Co. (Tokyo, Japan). Polyclonal antibodies against NF-κB subunits (p50 and p65) were from Santa Cruz Biotech, Inc. (Santa Cruz, Calif.). Glutathione ester, 7-aminoo-1-chloro-3-oxoliamido-2-heptanone (TLCK), and 1-t-lysylamido-2-phenylethyl chloromethyl ketone (TPCK) were from Sigma. Proto Blot kit (Western blot AP system kit) was from Promega Corp. (Madison, WI).

Cell Culture—The mouse hemangiendothelioma (MHE) cell line established from the thyroid, expressing factor VIII and vimentin (19), was used as vascular endothelial cells. MHE cells can incorporate acetylated low-density lipoprotein and stain positively for factor VIII antigen. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in 5% CO\(_2\) under 100% humidity. The cell cultures were centrifuged at 4 °C. The cell extract was prepared by lysis with 4 volumes of 10 mM Na\(_2\)PO\(_4\)/NaH\(_2\)PO\(_4\), pH 7.4, containing 0.5 mM EDTA, 0.1 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride, followed by sonication for 2 min. The cellular debris was removed by centrifugation at 4 °C for 60 min at 5,000 × g. The levels of γ-GCS and its related enzymes in the supernatant were estimated.

Enzyme Assay—The activity of γ-GCS was estimated using l-cysteine, α-amino butyric acid, and \(^{32}P\)ATP as substrates as described (20). The activities of glutathione peroxidase, glutathione S-transferase and Cu/Zn-superoxide dismutase (Cu/Zn-SOD) were estimated spectrophotometrically as described by Beutler (21). One unit of enzyme activity was expressed as 1 μmol of substrate changed per min.

Estimation of ATP and Glutathione—The concentrations of intracellular ATP, GSH, and GSSG were estimated enzymatically as described by Beutler (21).

Northern Blots—The cloned cDNA was isolated as described by Goldin et al. (7). A γ-GCS probe (267 base pairs corresponding to nucleotides 54–320 of rat kidney γ-GCS) was generated from rat liver γ-GCS cDNA and was used for cloning by the polymerase chain reaction. A human Cu/Zn-SOD cDNA fragment was generously provided by Dr. Taniguchi. For control hybridization on the amount of RNA, the complementary oligonucleotide fragment was hybridized to the RNA and Northern blotting were essentially as described by Sambrook et al. (23). Cytoplasmic RNAs isolated from MHE cells were subjected to electrophoresis in 1% agarose gels containing 0.6 M formaldehyde, stained with ethidium bromide, and then transferred to nylon membranes, and then hybridized with \(^{32}P\)labeled 28 S rRNA probe, and the intensity of the bands at 5.0 kilobases and 9.7 kilobases was measured. The relative radioactivity was expressed as a ratio of PSL corrected by the intensity of 28 S rRNA.

Electrophoretic Mobility Shift Assay—The electrophoretic mobility shift assay for NF-κB was performed as described by Sen and Baltimore (24) with slight modification. Briefly, nuclear extracts were incubated with an NF-κB-specific \(^{32}P\)-oligonucleotide. The binding reaction proceeded in a 20-μl volume containing 10 μg of extract, 4 μl of a binding buffer (10 mM Tris, pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 4% glycerol), 2 μg of poly(dI-dC) as a nonspecific competitor DNA, 2 μg of bovine serum albumin and labeled oligonucleotide (3,000–6,000 cpm). After a 30-min binding reaction at room temperature, samples were loaded on a 6% nondenaturing polyacrylamide gel and subjected to electrophoresis in 50 mM Tris, 45 mM borate, and 0.5 mM EDTA, pH 8.0. As specificity control, a 100-fold excess of unlabeled probe was applied. The sequence of the binding site for the NF-κB probe was 5′-GGGATTTCCTC-3′. The DNA-binding activity of the extract was quantitated by estimating the amount of the \(^{32}P\)-labeled NF-κB-DNA complex excised from the dried gels and was expressed as PSL.

RESULTS

Concentration of Glutathione and the Activity of Its Related Enzymes—Incubation of MHE cells with increasing concentrations of glucose resulted in a corresponding decrease in the concentration of GSH after 7 days. The concentration of GSH in 11, 22, and 28 mM glucose (Table I) was 78, 59, and 56% of that with 5.5 mM glucose, respectively. As shown in Table I, the activity of γ-GCS in cells incubated with 28 mM glucose was 51% of the activity in cells incubated with 5.5 mM glucose.

| Condition | GSH | ATP | γ-GCS | GST | Cu/Zn-SOD |
|-----------|-----|-----|-------|-----|-----------|
| 5.5 mM glucose | 11.7 ± 1.3 | 1.2 ± 0.1 | 2.53 ± 0.35 | 133.3 ± 2.4 | 211.8 ± 3.4 |
| 28 mM glucose | 6.5 ± 0.7* | 1.2 ± 0.1 | 1.28 ± 0.51* | 115.5 ± 1.6* | 210.0 ± 5.0 |

* p < 0.01 versus 5.5 mM glucose.

Table I

Concentration of glutathione and the activities of its related enzymes

Values are means ± S.D. of four experiments. GPX, glutathione peroxidase; GST, glutathione S-transferase.
Incubation. No stimulatory effect of IL-1
mine the effects of the cytokines were performed using a 3-h
a gradual decline (Fig. 1). The following experiments to deter-

Effects of Cytokines on the Concentration of Glutathione—
Incubating MHE cells with IL-1β or TNF-α resulted in a
marked increase in the concentration of GSH which reached a
maximum after 3 h of 547 and 474%, respectively, followed by
a gradual decline (Fig. 1). The following experiments to deter-
mine whether the high concentration of glucose
results in a decrease in the level of

Decrease in the activity of Cu/Zn-SOD by 69% (Fig. 2A).
A TNF-α receptor binding assay showed no apparent change in the characteris-
tics of the receptor between the two cell groups (data not
shown).

IL-1β and TNF-α also had stimulatory effects on the level
of activity of γ-GCS in cells grown in 5.5 mM glucose (172 and
192% increase compared with the control, respectively) but no
effect on the level of activity of γ-GCS in MHE cells incubated
with 28 mM glucose for 7 days (Fig. 2B). A TNF-α receptor
binding assay showed no apparent change in the characteris-
tics of the receptor between the two cell groups (data not
shown).

This suggested that impairment of the response to TNF-α
cells exposed to high glucose concentration is not due to
changes in the receptor levels.

Northern Blots—Effects of cytokines and glucose concentration
on the expression of γ-GCS mRNA were estimated by
Northern blotting. IL-1β and TNF-α stimulated the expression of
γ-GCS mRNA (Fig. 3). TNF-α and IL-1β increased the
expression of γ-GCS mRNA by 225 and 220%, respectively. Incu-
bating MHE cells treated with 28 mM glucose for 7 days re-
sulted in a decrease in the level of γ-GCS mRNA by 30% (lane
4), and the cytokine addition did not affect the expression of
γ-GCS mRNA under these conditions. The results indicate that
IL-1β and TNF-α stimulate the expression of γ-GCS mRNA
followed by concomitant increases in the γ-GCS activity and

Fig. 1. Time course study on the effect of cytokines on the
concentration of GSH. MHE cells were incubated with 100 units/ml
of IL-1β (○) or 100 units/ml of TNF-α (●), and changes in intracellular
GSH were estimated. Values are means ± S.D. of four experiments.

Fig. 2. Effect of high glucose concentration on the concentra-
tion of glutathione and the activity of γ-glutamylcysteine synthetase
stimulated by cytokines in endothelial cells. MHE cells were
incubated with 5.5 or 28 mM glucose for 7 days, then the effects of
IL-1β (100 units/ml) or TNF-α (100 units/ml) after 3 h on the GSH
concentration (A), and the γ-GCS activity (B) was estimated. Unfilled
bars represent cells incubated with 5.5 mM glucose and filled bars are
those with 28 mM glucose. Values are means ± S.D. of four experiments.

GSH concentration and that exposing MHE cells to a high
concentration of glucose negates the stimulatory effect of these
cytokines.

Activity of NF-κB—The effect of TNF-α on the expression of
NF-κB was estimated using a gel-shift assay. The concentra-
tion of NF-κB was increased 5-fold when MHE cells with 5.5
mM glucose were incubated with TNF-α (Fig. 4). Unfilled bars
represent cells incubated with 5.5 mM glucose and filled bars are
those with 28 mM glucose. Values are means ± S.D. of four experiments.
Effect of Glucose on the Expression of \(\gamma\)-Glutamylcysteine Synthetase

Fig. 3. Northern blots of \(\gamma\)-glutamylcysteine synthetase mRNA in endothelial cells. After incubating MHE cells with or without cytokines for 3 h, about 30 \(\mu\)g of each total RNA extracted from the cells was fractionated by electrophoresis through 1% agarose, transferred to nylon membranes, and hybridized with \(^{32}\)P-labeled nick-translated \(\gamma\)-GCS DNA (A). Lanes 1–3, cells incubated with 5.5 mM glucose; lane 2, + TNF-\(\alpha\); lane 3, + IL-1\(\alpha\); lanes 4–6, cells incubated with 28 mM glucose; lane 5, + TNF-\(\alpha\); lane 6, + IL-1\(\alpha\), as described in the legend to Fig. 2. The relative amounts of the radioactivity are expressed as PSL (B). Each numbered bar corresponds to the lane in A. Data are means \pm S.D. of three independent analyses.

Fig. 4. Electrophoretic mobility shift assay of NF-\(\kappa\)B. The DNA binding activity of NF-\(\kappa\)B was estimated using an electrophoretic mobility shift assay. MHE cells were incubated with 5.5 or 28 mM glucose for 7 days and then for 3 h in the presence or absence of 100 units/ml of TNF-\(\alpha\). DNA extracts from each of these cells were incubated with a NF-\(\kappa\)B-specific \(^{32}\)P-digoxigenin-labeled DNA fragment for 30 min and then loaded on to a 6% nondenaturing polyacrylamide gel. For specificity controls, a 100-fold excess of unlabeled probe was applied (lane 1). Lanes 2 and 3, cells incubated with 5 mM glucose; lanes 4 and 5, cells incubated with 28 mM glucose; lanes 3 and 5, + TNF-\(\alpha\), as described in the legend to Fig. 2. Positions of specific DNA-protein complexes are indicated by an arrowhead.

suggest that the inhibition of NF-\(\kappa\)B activation inhibits the TNF-\(\kappa\)-dependent expression of \(\gamma\)-GCS mRNA.

To obtain additional evidence for this suggestion, we incubated MHE cells with the antisense codons for p50 and p65. Adding the antisense codons 24 h before TNF-\(\alpha\) inhibited TNF-\(\kappa\)-dependent increase in TNF-\(\kappa\)B concentration (Fig. 5A, lane 6) and the expression of \(\gamma\)-GCS mRNA stimulated by TNF-\(\alpha\) (Fig. 6, lanes 7 and 8). An immunological analysis of cell extracts from MHE cells indicated that the concentration of both p50 and p65 proteins increased by TNF-\(\alpha\) and that the authentic antisense codons inhibited the synthesis of these proteins (Fig. 5B).

Nuclear Run-on Assay—We performed nuclear run-on transcriptional assays to determine whether the decreased expression of the \(\gamma\)-GCS mRNA in the presence of high glucose concentration was due to altered transcriptional activity of this gene. Shift of the concentration of glucose to 28 mM decreased the transcriptional rates of \(\gamma\)-GCS by 58% (Fig. 7, lanes 1 and 3). Its transcriptional rates in response to TNF-\(\alpha\) were 6.0-fold higher in MHE cells incubated with 5.5 mM glucose (lanes 1 and 2) and only 1.1-fold in the presence of 28 mM glucose (lanes 3 and 4). The transcriptional rates of Cu,Zn-SOD in 28 mM glucose slightly changed by 90% compared with that in 5.5 mM glucose, and TNF-\(\alpha\) had no apparent effect on the transcription of Cu,Zn-SOD in both glucose concentrations (lanes 2 and 4).

Modification of Glutathione Levels—The effect of the changes in the concentrations of GSH and GSSG on the expression of \(\gamma\)-GCS mRNA was studied. As shown in Table I, when the cells incubated with 5.5 mM glucose were exposed to 1 mM GSH ester 48 h before analysis, the concentration of GSH and the expression of \(\gamma\)-GCS mRNA increased by 163 and 141%, respectively. GSH ester also increased the GSH/GSSG ratio by 170%. When the cells incubated with 28 mM glucose, in addition to the decrease in the concentration of GSH, the concentration of GSSG increased by 146%, and the GSH/GSSG ratio decreased by 33%. GSH ester added to the cells incubated with 28 mM glucose increased the concentration of GSH by 266% and the expression of \(\gamma\)-GCS mRNA by 283%, decreased the concentration of GSSG by 78%, and increased the GSH/GSSG ratio by 343%, respectively. Furthermore, it restored the weakened response of \(\gamma\)-GCS mRNA to TNF-\(\alpha\) as well as to IL-1\(\beta\). These data suggest that the expression of \(\gamma\)-GCS mRNA correlates with the concentration of GSH more than with the concentration of GSSG or the GSH/GSSG ratio.

DNA Damage—To establish if DNA from cells incubated with a high concentration of glucose is sensitive to oxidative...
Effect of Glucose on the Expression of \( \gamma \)-Glutamylcysteine Synthetase

**Fig. 6.** Effect of NF-\( \kappa \)B inhibition on the expression of \( \gamma \)-GCS. A, Northern blots. The effect of protease inhibitors and antisense codons of \( \gamma \)50 and \( \gamma \)65 on the TNF-\( \kappa \)B-stimulated expression of \( \gamma \)-GCS mRNA was studied by Northern blotting. Lane 1, control; lane 2, \( \kappa \)50; lane 3 and 4, \( \kappa \)300 \( \mu \)M TLCK; lane 5 and 6, \( \kappa \)300 \( \mu \)M TPCK; lane 7 and 8, + antisense codons of \( \gamma \)50 and \( \gamma \)65. B, the relative amounts of the radioactivity are expressed as PSL. Each lane corresponds to that in A.

stress, DNA damage by oxidative stress was studied by estimating the formation of 8-OHdG (Fig. 8). Nucleosomes prepared from MHE cells incubated with 5.5 \( \mu \)M glucose for 7 days showed no apparent increase in the levels of 8-OHdG after adding 25 \( \mu \)M tert-butylhydroperoxide for 4 h. In contrast, nucleosomes from cells incubated with 28 \( \mu \)M glucose showed an increase in the level of 8-OHdG induced by 25 \( \mu \)M tert-butylhydroperoxide (5.8 \pm 0.4 pmol of 8-OHdG/g of DNA with 28 \( \mu \)M glucose versus 0.6 \pm 0.1 pmol of 8-OHdG/g of DNA with 5.5 \( \mu \)M glucose). This DNA damage due to 25 \( \mu \)M tert-butylhydroperoxide in MHE cells with 28 \( \mu \)M glucose was restored to normal levels when cells were incubated beforehand with 1 \( \mu \)M GSH ester for 48 h.

**DISCUSSION**

To maintain cellular functions, relatively high concentrations of GSH are maintained through synthesis, redox cycle supported by GSH reductase and GSH peroxidase, and the active transport of GSSG or GSH S-conjugates. In particular, \( \gamma \)-GCS regulates the overall turnover of the intracellular GSH (4). The activity of \( \gamma \)-GCS is regulated by the nonallosteric inhibition by GSH (5). Moreover, the levels of GSH are regulated by the expression of \( \gamma \)-GCS. An increase in GSH concentration caused by elevated transcriptional levels of \( \gamma \)-GCS has been reported (7, 34). GSH synthesis in response to various stresses occurs rapidly (9). The rapid response of the expression of \( \gamma \)-GCS mRNA to heat shock or oxidative stress is followed by corresponding elevations of the concentration of GSH, demonstrating the important function of GSH and \( \gamma \)-GCS in cellular emergencies. We have reported that depletion of GSH causes embryogenesis in rat embryo culture (35). The decreased expression of \( \gamma \)-GCS mRNA in rat embryos cultured with a high concentration of glucose was reversed when the embryos were exposed to GSH ester, suggesting that oxidative conditions alter the expression of \( \gamma \)-GCS mRNA.

Endothelial cells face the circulating blood. An increase in cytotoxicity caused by \( \text{H}_2\text{O}_2 \) was found in endothelial cells cultured in high concentrations of glucose (36), and a weakened defense system against oxidative stress is thought to contribute to the development of diabetic complications. In this study, the exposure of endothelial cells to a high concentration of glucose for 7 days decreased the expression of \( \gamma \)-GCS mRNA (Fig. 3), followed by a concomitant decrease in the enzyme protein, the enzyme activity, and the concentration of GSH (Table I, Fig. 2), in good agreement with a previous report (8).
IL-1β and TNF-α have ubiquitous biological activities and play diverse biological roles in host systems. They induce chronic inflammatory changes and bring about increases in a variety of defense mechanisms, particularly immunologic and hematologic responses (2). Endothelial cells synthesize IL-1β and TNF-α and have specific receptors for them. Kalebic et al. have reported (37) that expression of the human immunodeficiency virus in chronically infected monocyte cells is induced by TNF-α and suppressed by GSH, suggesting a relationship between cellular GSH and the viral infection mediated by TNF-α. A protective effect of IL-1β against oxidative stress has been reported by Tsan et al. (38). Administering IL-1β to rats increased the activities of pulmonary Mn-SOD, and Cu,Zn-SOD. However, the effects of these cytokines on the defense systems in endothelial cells have not been investigated in detail.

This results here represent the first report that TNF-α stimulates the expression of γ-GCS, resulting in an increase in the levels of GSH. The concentration of GSH in endothelial cells increased within 3 h after the addition of TNF-α or IL-1β and gradually returned to the initial level after 48 h (Fig. 1). This increase in the concentration of GSH was correlated with an increase in the expression of γ-GCS mRNA and a corresponding increase in the activity of this enzyme (Figs. 2 and 3). Marcho et al. (39) have reported a decrease in the concentration of GSH and an increase in the susceptibility to oxygen toxicity in bovine pulmonary artery endothelial cells treated with TNF-α for 18 h. Similar observations were reported by Ishii et al. (40) who found that TNF-α administration for 6 h mediates a decrease in the concentration of GSH and an increase in the sensitivity of pulmonary vascular endothelial cells to H₂O₂. The reason for the difference in the response between pulmonary endothelial cells reported by Ishii et al. (40) and the results here is unclear.

NF-κB is inducible both by IL-1β and TNF-α (41, 42). Simultaneous activation of NF-κB and the expression of γ-GCS by TNF-α suggest that the effect of TNF-α on the γ-GCS mRNA is mediated by NF-κB (Fig. 4). Proteolytic steps of IκB are important in the activation of NF-κB (13). The protease inhibitors, TLCK and TPCCK, inhibited the TNF-α-dependent activation of NF-κB and the expression of γ-GCS mRNA (Figs. 5 and 6). The expression of γ-GCS mRNA was similarly inhibited when NF-κB was blocked by its antisense codons (Fig. 6). These results suggest that NF-κB initiates expression of γ-GCS. The result of the nuclear run-on assay for the expression of γ-GCS stimulated by TNF-α indicates that the transcriptional levels of γ-GCS are regulated by TNF-α (Fig. 7), whereas those of Cu,Zn-SOD are not in good agreement with the data reported by Visner et al. (30).

The endothelial cells exposed to high concentration of glucose for 7 days showed a decrease in the expression of γ-GCS mRNA and its response to TNF-α and IL-1β (Figs. 2–4). NF-κB is regulated by oxidoreductive control mechanisms (43), and the oxidoreductive condition of NF-κB regulates its DNA binding activity (44). Galter et al. (45) have reported the inhibitory effect of GSSG on the DNA binding activity of NF-κB activation.

A striking observation in this study is that elevation of the concentration of GSH by the addition of GSH ester to the cells incubated with 28 mM glucose resulted in an increase in the expression of γ-GCS. Furthermore, it restored the weakened response of the expression of γ-GCS mRNA to TNF-α and IL-1β (Table I). The effect of modification of GSH metabolism on the increase in the expression of γ-GCS was apparently paralleled by an increase in the concentrations of GSH. These observations suggest that GSH concentration is important in the regulation of the expression of γ-GCS and the impaired expression of γ-GCS in the cells incubated with a high glucose concentration causes changes in the redox regulation system. However, the precise mechanisms by which this responsiveness of γ-GCS is impaired in the cells remain to be clarified.

Since endothelial cells, which express insulin-independent glucose transporter-1, are exposed to intracellular high glucose concentration levels in diabetes mellitus, we propose that a weak response of GSH synthesis to cytokines together with low levels of GSH, reduces cellular antioxidant activity, and is a potential cause of endothelial cell damage and the development of diabetic complications. This is supported by the findings in this study that the DNA damage is increased by oxidative stress in the endothelial cells exposed to high concentrations of glucose (Fig. 8).

In conclusion, long exposure of endothelial cells to high glucose levels impairs GSH synthesis stimulated by cytokines, which apparently weakens defense systems against oxidative stress in diabetes mellitus.

**Fig. 8.** DNA damage by oxidative stress. DNA damage was estimated using nucleosomes prepared from MHE cells incubated with 28 mM glucose (A) or 5.5 mM glucose (B) for 7 days. Nucleosomes prepared from each group were treated with 25 μM tert-butylhydroperoxide in vitro for 4 h, and formation of 8-OHdG was estimated. Arrow indicates the peak fraction of the internal standard for 8-OHdG.
REFERENCES

1. Furchgott, R. F., and Zawadzki, J. V. (1980) Nature 285, 373–376
2. Stocker, R., Hunt, N. H., Buffinton, G. D., Weidemann, M. J., Lewis-Hughes, P. H., and Clark, I. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 548–551
3. Halliwell, B., and Gutteridge, J. M. C. (1984) Lancet 1, 1396–1397
4. Meister, A. (1985) Methods Enzymol. 113, 571–585
5. Richman, P., and Meister, A. (1975) J. Biol. Chem. 250, 1422–1426
6. Yan, N., and Meister, A. (1985) Science 228, 759–763
7. Goldin, A. K., Meister, A., and Baltimore, D. (1988) Mol. Cell. Biol. 89, 705–716
8. Tagami, S., Kondo, T., Yoshida, K., Hirokawa, J., Ohtsuka, Y., and Kawakami, Y. (1990) Metabolism 39, 1033–1038
9. Kondo, T., Yoshida, K., Urata, Y., Goto, S., Gasas, S., and Taniguchi, N. (1993) J. Biol. Chem. 268, 20366–20372
10. Vilcek, J., and Lee, T. H. (1991) J. Biol. Chem. 266, 7313–7316
11. Dinarello, C. A. (1991) Blood 77, 1627–1652
12. Mandrup-Poulsen, T., Bendtz, K., Dinarello, C. A., and Nerup, J. (1987) J. Immunol. 139, 4077–4082
13. Finco, T. S., Beg, A. A., and Baldwin, A. S., Jr. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11884–11888
14. Baeverl, P. A., and Baltimore, D. (1988) Science 242, 540–546
15. Nakayama, K., Shimo, H., Mitomo, K., Watanabe, T., Okamoto, S. I., and Yamamoto, K. (1992) Mol. Cell. Biol. 12, 705–716
16. Cauley, K., and Verma, I. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 390–394
17. Liou, H. C., and Baltimore, D. (1993) Curr. Opin. Cell Biol. 5, 477–487
18. Baeverl, P. A. (1991) Biochim. Biophys. Acta 1072, 63–80
19. Hosoda, C., Ohtsuru, A., Namiba, H., Motomura, K., Hara, T., Nakashima, M., Ito, M., Yokos, K., and Yamashita, S., (1995) Endocrinology 32, 342–350
20. Beutler, E., and Gelbart, T. (1986) Clin. Chim. Acta 158, 115–123
21. Beutler, E. (1984) in Red Cell Metabolism (Beutler, E., ed) 3rd Ed., pp. 77–78, Grune & Stratton, New York
22. Barbu, V., and Dautry, F. (1988) Nucleic Acids Res. 17, 7115
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 7–39, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Sen, R., and Baltimore, D. (1986) Cell 46, 705–716
25. Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P., and Baltimore, D., (1990) Cell 62, 1019–1029
26. Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeverl, P. A., and Rosen, C. A. (1991) Science 251, 1490–1493
27. Wu, G. Y., and Wu, C. H. (1992) J. Biol. Chem. 267, 12436–12439
28. Redinbaugh, M. G., and Turley, R. B. (1986) Anal. Biochem. 153, 267–271
29. Hamaoka, R., Keho, K., Seguchi, T., Okamura, K., Morimoto, A., Ono, M., Ogata, J., and Kuwano, M. (1992) J. Biol. Chem. 267, 13160–13165
30. Visner, G. A., Dougl, W. C., Wilson, J. M., Burr, I. A., and Nick, H. S. (1990) J. Biol. Chem. 265, 2856–2864
31. Manji, S. S., Zhou, H., Findlay, D. M., Martin, T. J., and Ng, K. W. (1995) J. Biol. Chem. 270, 8958–8962
32. Kasai, H., Crain, P. F., Kudoh, Y., Nishimura, S., Ottsuyama, A., and Tanokka, H. (1996) Carcinogenesis 17, 1849–1851
33. Muramatsu, M., Hayashi, T., Onishi, T., Takai, K., and Kashiwaya, T. (1974) Exp. Cell Res. 88, 345–351
34. Goto, S., Yoshida, K., Morikawa, T., Urata, Y., Suzuki, K., and Kondo, K. (1996) Cancer Res. 55, 4297–4301
35. Trocino, A. T., Akazawa, S., Ishibashi, M., Matsumoto, K., Matsu, H., Yamamoto, H., Goto, S., Urata, Y., Kondo, T., and Nagata, S. (1995) Diabetes 44, 1–7
36. Kashiwagi, A., Asahina, T., Ikebuchi, Y., Tanaka, Y., Takagi, Y., Nishio, Y., Kikukawa, R., and Shigeta, Y. (1994) Diabetologia 37, 264–269
37. Kaledic, T., Kinter, A., Pali, G., Anderson, M. E., Meister, A., and Fauq, A. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 88, 886–900
38. Tsn, M. F., Lee, C. Y., and White, J. E. (1991) J. Appl. Physiol. 71, 688–697
39. Mando, Z., White, J. E., Higgins, P. J., and Tsn, M. F. (1991) Am. J. Respir. Cell Mol. Biol. 5, 556–562
40. Ishii, Y., Partridge, C. A., Vecchio, J. D., and Malik, A. B. (1992) J. Clin. Invest. 89, 794–802
41. Stylianou, E., O'Neil, L. A. J., Martin, T. J., and Ng, K. W. (1995) J. Biol. Chem. 268, 18018–18029
42. Visner, G. A., Dougl, W. C., Wilson, J. M., Burr, I. A., and Nick, H. S. (1990) J. Biol. Chem. 265, 2856–2864
43. Joshi-Barve, S., Rangnekar, V. V., Sells, S. F., and Rangnekar, V. M. (1993) J. Biol. Chem. 268, 13160–13165
44. Toleno, M. B., and Leonard, W. J. (1990) J. Biol. Chem. 265, 15836–15841
45. Galter, D., Mihm, S., and Droge, W. (1994) Eur. J. Biochem. 221, 639–648
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Yoshishige Urata, Hidefumi Yamamoto, Shinji Goto, Hideki Tsushima, Shouichi Akazawa, Shunichi Yamashita, Shigenobu Nagataki and Takahito Kondo

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