Increase in Thioredoxin Activity of Intestinal Epithelial Cells Mediated by Oxidative Stress

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Hydrogen peroxide was cytotoxic to the small intestine epithelial cell line, IEC-6, as judged from an MTT assay and the release of lactate dehydrogenase. The glutathione S-transferase and thioredoxin reductase activities and SH content decreased dose-dependently with H_2O_2, but thioredoxin activity increased at low H_2O_2 concentrations. In addition, the increase in thioredoxin activity was time-dependent during the initial stages of oxidative stress. A reverse transcription-polymerase chain reaction (RT-PCR) amplification also showed that the mRNA content in IEC-6 cells increased time-dependently at 0.25 mM H_2O_2. These results indicate that cellular oxidative shock causes an increase in the activity of thioredoxin, which is involved in the defense mechanism against oxidative stress.

Key words thioredoxin; oxidative stress; IEC-6; H_2O_2

There are many opportunities for damage to the small intestine resulting from exposure to stress by xenobiotics, bacteria, medicines, and heavy metals. Additionally, there is increasing interest in the possible role of reactive oxygen radicals in intestinal diseases. Oxygen species have been implicated in ischemia-induced permeability changes of the intestine, and in causing intestinal inflammation.[1,2] The ubiquitous nature of radical generation has required all aerobic organisms to develop numerous intracellular and extracellular detoxification mechanisms.[3] Thioredoxin is a small, ubiquitously expressed protein, originally discovered as a hydrogen donor for ribonucleotide reductase[4] and subsequently implicated in a variety of biochemical pathways.[5] Thioredoxin functions as an efficient cytosolic antioxidant and is found in all eukaryotic and prokaryotic cells.[5] It has so far remained largely uncharacterized in the small intestine, although we have previously characterized the thioredoxin and thioredoxin reductase of rat small intestine.[6] In the present study, we focused on thiol-related enzymes, including thioredoxin and thioredoxin reductase, in a small intestine epithelial cell line, IEC-6 monolayers. We selected hydrogen peroxide as a means of imparting oxidative stress, and examined its time- and dose-dependent effect on the cells.

MATERIALS AND METHODS

Enzymes and Cells Thioredoxin reductase of rat liver was purified by the method of Luthman and Holmgren[7] with a slight modification. The cell line IEC-6 was a kind gift from Dr. K. Tanaka, Osaka University, Japan. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% fetal bovine serum (Gibco) in an atmosphere of 5% CO_2 and room air at 37 °C. The culture medium was changed every 3 d. Monolayers with 90% confluence were subcultured by trypsinization using 0.25% trypsin (Difco Laboratories) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. Cultures were examined under an inverted light microscope on a regular basis to monitor growth and contamination.[8]

Enzyme Assays Cells seeded onto 90ϕ dishes (Sumilon) at 2×10^4 cells/ml and cultured for 18 h were incubated with or without H_2O_2 (Santoku Chemicals) in 7 ml serum-free defined medium for the indicated periods. The cells were then washed with phosphate buffered saline and suspended in 1 ml phosphate buffered saline by scraping with a rubber-tipped spatula. The cell suspension was homogenized with a hand-held micro-homogenizer (Physcotron) and centrifuged at 20000×***g*** for 10 min. Activities of the following enzymes in the supernatant were measured as described previously: thioredoxin reductase,[9] glutathione S-transferase[10] and lactate dehydrogenase.[11] Thioredoxin activity was determined by measuring the reduction of insulin in the presence of thioredoxin reductase, and NADPH.[9] After incubation for 30 min at 37 °C, the reaction was terminated by adding 6.4 M urea, and free SH groups were determined colorimetrically by the method of Ellman.[12] The reaction carried out without NADPH was used as the control. One unit of thioredoxin was defined as such quantity which increased the absorbance at 412 nm by 0.1 after 30 min incubation at 37 °C.

MTT Assay and SH Content The MTT assay was performed as described by Mosmann[13] with a slight modification. Cells seeded onto 24-well plates (Sumilon) at 2×10^4 cells/ml and cultured for 18 h were incubated with or without H_2O_2 in a total of 400 μl of serum-free defined medium for the indicated periods. One milliliter of 3-(4,5-dimethyl-thiazole-2-yl) 2,5-diphenyl tetrazolium bromide (MTT, Wako Pure Chemicals) solution (1 mg/ml) was added to all of the wells, followed by incubation at 37 °C for 4 h. Finally, an aliquot of 100 μl dimethyl sulfoxide was added to all the wells and the solution was mixed to dissolve the formazan of the MTT. The absorbance of the solution at 540 nm was then measured. The SH content was estimated spectrophotometrically using 50 mM 5,5′-dithiobis (2-nitro-benzoate) (Wako Pure Chemicals) in 1% SDS and 0.1 M Tris–HCl, pH 8.0, with reduced glutathione (Wako Pure Chemicals) as a reference.

RT-PCR Amplification of the Thioredoxin Transcript Total RNA was extracted from IEC-6 cells and the genomic

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DNA was digested by Isogen (Nippon Gene). Sense and antisense primers of thioredoxin were synthesized (BEX) in the following sequences. Sense: ATCCATTTCCATCGGGTTCTGC; antisense: GCAGTTGGGTATAGACTCTC. Total RNA samples of 1 μg were subjected to RT-PCR amplification using a RT-PCR high-Plus-kit (Toyobo) under the conditions of 15 cycles at 94 °C for 1 min, and 60 °C for 1.5 min. The same amplification in the total RNA samples was carried out using glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers which were included in the RT-PCR high-Plus-kit. The amplified products were electrophoresed on a 1% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining. The RT-PCR products of G3PDH were employed as an internal control.

Protein Assay The protein concentration was measured using a protein assay kit purchased from BioRad.

RESULTS AND DISCUSSION

Active oxygen species, including H_2O_2, are produced by activated neutrophils or xanthine oxidase. To investigate the H_2O_2-induced cytotoxicity of the intestine, we used the small intestine epithelial cell line IEC-6. As shown in Fig. 1, the cytotoxicity of IEC-6 cells was revealed by the MTT assay to be dependent on the H_2O_2 concentration and the duration of the incubation. IEC-6 cells exhibited no cytotoxicity after 8-h incubation with 0.25 mM H_2O_2, but the cells were susceptible to a dose at a concentration of 1 mM. At this concentration of H_2O_2, the cell damage after 6-h incubation was roughly equivalent that after 8-h. Therefore, the incubation of IEC-6 cells for 6 h was considered a sufficient length of time to observe the cytotoxic effect of H_2O_2.

To clarify the cytotoxicity arising from oxidative stress, decreases in the cellular SH content and glutathione S-transferase activity were monitored as described by Shen *et al.*[14] As shown in Fig. 2(a), these decreases were dependent on the H_2O_2 concentration, reflecting the oxidative damage to the IEC-6 cells. At 0.25 mM H_2O_2, the thioredoxin activity in the cells had slightly increased after 6-h incubation, while the SH content and the glutathione S-transferase and thioredoxin reductase activities retained their initial levels, in spite of the oxidative shock. This suggests that cellular thioredoxin plays a protective role against oxidative stress by elevating its activity. In the presence of NADPH, thioredoxin reductase is needed for the reduction of oxidized thioredoxin, but activation of the enzyme was not observed. As shown in Fig. 2(b), the release of lactate dehydrogenase from IEC-6 cells occurred during incubation in the presence of H_2O_2; however, at 0.25 mM H_2O_2 no release of the enzyme was observed, reflecting the preventive effect of thioredoxin against oxidative stress.

After incubating IEC-6 cells with serum-free DMEM for 6, 12, and 24 h in the presence of 0.25 mM H_2O_2, the thioredoxin activity in the cell lysate was measured. There were 1.5-, 8.4-, and 8.6-fold increases in thioredoxin activity after 6-, 12-, and 24-h incubation, respectively (Fig. 3). Since there is a possibility that oxidative shock in IEC-6 cells resulting from H_2O_2 will cause the adaptive induction of the thioredoxin protein in the cells, Western blotting was employed to analyze the cell thioredoxin. However, the extremely small amounts of the protein did not afford positive

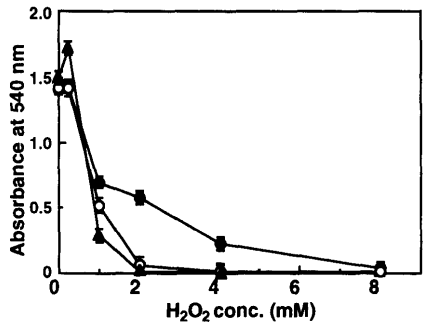

Fig. 1. Dose-Dependent Effect of H_2O_2 on IEC-6 Cell Survival

Cells were incubated with 0—8 mM H_2O_2 for 4 (●), 6 (○), or 8 (▲) h. Bars represent the means±S.D. of three experiments.

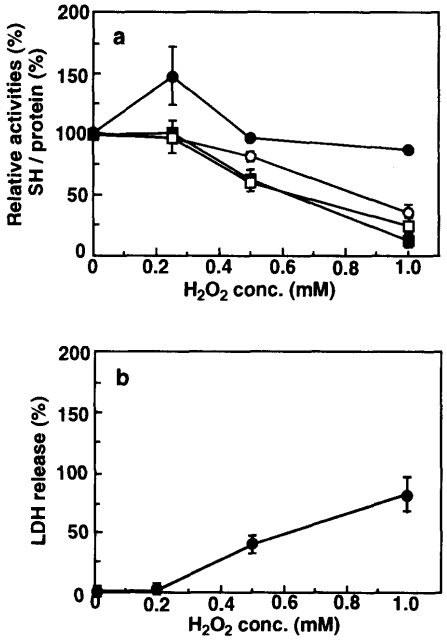

Fig. 2. (a) Effect of H_2O_2 on Thioredoxin (Txn), Thioredoxin Reductase (ThRD), Glutathione S-Transferase (GST) Activities and Thiol Content in IEC-6 Cells

IEC-6 cells were treated with 0—10 mM H_2O_2 and cell lysates were prepared as described in Materials and Methods. The specific activities of Txn (●), ThRD (○), GST (■), and the thiol content (□) are expressed as a percentage of the values obtained in cells not treated with H_2O_2. (b) Lactate dehydrogenase (LDH) released from damaged cells. The percentage of LDH release was defined as the ratio of LDH activity in the medium to the total activity per dish. Bars represent the means±S.D. of three experiments.

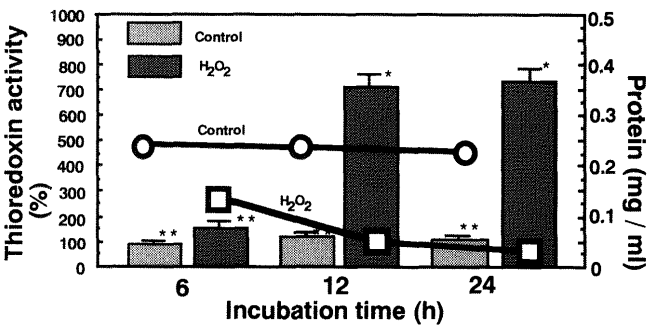

Fig. 3. Increases in Thioredoxin Activity in IEC-6 Cells Treated with H_2O_2

IEC-6 cells were treated in the presence of 0.25 mM H_2O_2 for 6, 12, or 24 h and in the absence of H_2O_2 as a control. The thioredoxin activity in the IEC-6 lysate was then measured and expressed as a percentage of that in untreated cell lysate. Bars represent the means±S.E. of two experiments. Asterisks indicate significant differences from 0 h (*, $p<0.05$; **, $p<0.01$). The protein concentrations were concomitantly measured in the lysate of IEC-6 cells treated with H_2O_2 (□) and in the control (○).

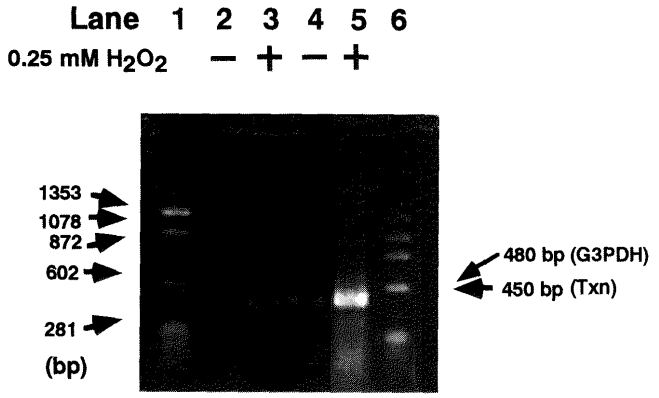

Fig. 4. Electrophoretic Analysis of the RT-PCR Products of Thioredoxin Transcripts in IEC-6 Cells Incubated with H_2O_2

Total RNA was extracted from IEC-6 cells treated in the presence or absence of 0.25 mM H_2O_2 for 6 h, and the total RNAs (1 μg/ reaction tube) were employed for PCR amplification with reverse transcription. Primers used for the amplification were as follows: lanes 4 and 5, thioredoxin ; lanes 2 and 3, G3PDH. Lanes 1 and 6 are molecular weight markers of ϕX174/*Hae* III digest.

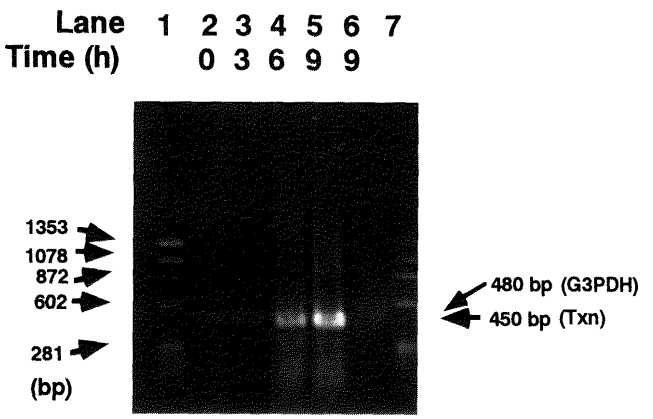

Fig. 5. Effect of H_2O_2 on Thioredoxin mRNA Expression in IEC-6 Cells after Treatment for Various Lengths of Time

Total RNA was extracted from IEC-6 cells treated in the presence of 0.25 mM H_2O_2 for 0, 3, 6, and 9 h. The total RNAs (1 μg/ reaction tube) were employed for PCR amplification with reverse transcription. Primers used for the amplification were as follows: lanes 2, 3, 4, and 5, thioredoxin ; lane 6, G3PDH. Lanes 1 and 7 are molecular weight markers of ϕX174/*Hae* III digest.

results, and further analysis will be required. The fact that very low amounts of protein were recovered after 12- and 24-h incubation with H_2O_2 reflects cell lysis under oxidative stress, as depicted in Fig. 3.

After incubating IEC-6 cells with 0.25 mM H_2O_2 for 6 h, total RNA was isolated from the cells and 1 μg samples were subjected to RT-PCR amplification analysis. An increase was observed in the level of the transcript for thioredoxin, but there was no increase in the housekeeping gene of G3PDH (Fig. 4). This finding showed that the induction of thioredoxin gene expression occurred in IEC-6 cells treated with H_2O_2. Total RNA was also isolated from IEC-6 cells at different times following treatment with 0.25 mM H_2O_2 (Fig. 5). The thioredoxin mRNA levels showed significant increases after 6 and 9 h, but not after 3 h, reflecting a time lag in the induction of thioredoxin gene expression.

Studies on antioxidant enzymes in the small intestine relating to scavengers of reactive oxygen species like superoxide dismutase or catalase have been reported.[15,16] Enzyme candidates in the cytosol able to eliminate the H_2O_2 produced by superoxide dismutase in the process of scavenging the superoxide anion need to be identified. Glutathione peroxidase is one of the candidates in the small intestine, but its activity there[15] is 25 times lower than in the liver.[17] On the other hand, thioredoxin activity in rat small intestine was found to be significant, even though its activity is half that in rat liver (Mizoguchi *et al.*, unpublished data). Catalase is localized in peroxisomes, so it is thought to eliminate H_2O_2 produced in these microbodies. There are only a few reports in the literature concerning the oxidative inactivation of intestinal enzymes, and none on the restoration processes of inactivated enzymes mediated by oxidative stress in the small intestine. The findings from the present work strongly suggest that thioredoxin localized in cytosol plays a role in protecting sensitive enzymes in the small intestine through the induction of thioredoxin gene expression and the stimulation of the protein activity. In fact, thioredoxin exhibits reducing activity against reactive oxygen[18] and restorative activity toward damaged proteins.[19—21] There have been several reports on the induction of thioredoxin in tissues or cells[22—24] but not in the digestive ducts, including the small intestine, which point to increased thioredoxin expression.

We are now planning to investigate details of the adaptive induction of thioredoxin and to further analyze the protein using Western blotting.

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