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Differential Thioredoxin Reductase Activity from Human Normal Hepatic and Hepatoma Cell Lines

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Thioredoxin reductase (TrxR), a component of the thioredoxin system, including thioredoxin (Trx) and NADPH, catalyzes the transfer of electrons from NADPH to Trx, acts as a reductant of disulfide-containing proteins and participates in the defense system against oxidative stresses. In this study, the regulation pattern of TrxR in the presence of various stressful reagents was compared between Chang (human normal hepatic cell) and HepG2 (human hepatoma cell) cell lines. Aluminum chloride (0.5 mM) and zinc chloride (0.5 mM) enhanced the TrxR activity in the Chang cell line to a higher degree than in the HepG2 cell line, but cupric chloride (0.2 mM) and cadmium chloride (0.1 mM) enhanced the TrxR activity in the HepG2 cell line to a greater degree. The TrxR activities in both Chang and HepG2 cell lines were similarly induced by treatment with sodium selenite (0.02 mM) and menadione (0.5 and 1.0 mM). Lipopolysaccharide (2 μg/ml) increased the TrxR activity upto 4.02- and 2.2-fold in the Chang and HepG2 cell lines, respectively, in time-dependent manners. Hydrogen peroxide (5 mM) markedly enhanced the TrxR activity in the HepG2 cell line, but not in the Chang cell line. NO-generating sodium nitroprusside (3.0 and 6.0 mM) induced TrxR activities in both human liver cell lines. The TrxR activity was also induced in human liver cells under limited growth conditions by serum deprivation. These results imply that the TrxR activities in normal hepatic and hepatoma cell lines are subject to different regulatory responses to various stresses.

Key Words: Chang, HepG2, human liver cell line, nitric oxide, oxidative stress, regulation, thioredoxin reductase

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INTRODUCTION

Thioredoxin reductase (TrxRs; EC 1.6.4.5) is a selenium-containing pyrimidine nucleotide-disulfide oxidoreductase, of the homodimeric flavoenzymes family, which also includes lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase.[1] TrxRs catalyze the NADPH-dependent reduction not only of the redox protein thioredoxin (Trx), but also of other endogenous and exogenous compounds.[2] Lipoic acid, lipid hydroperoxides, the cytotoxic peptide NK-lysin, vitamin K3, dehydroascorbic acid, the ascorbyl free radical and the tumor-suppressor protein p53 have been demonstrated to be endogenous substrates of TrxRs.[3-9]

Mammalian TrxR has a wide substrate specificity and directly reduces different protein disulfides, many low-molecular-weight disulfide compounds and nondisulfide compounds. This broad substrate specificity is largely based on a second redox-active site, a C-terminal -Cys-SeCys-Gly- (where SeCys is selenocysteine), which is not found in glutathione reductase or *Escherichia coli* TrxR.[10-13] The carboxy-terminal selenocysteine of mammalian TrxR is also likely to act as a cellular redox sensor.

As one of the major disulfide reductases, which regulate the cellular redox state and cell proliferation, possibly contributing to the drug resistance of malignant cells, the various physiological roles of TrxR have been reported in tumor cells. The expression of TrxR is significantly increased in some tumors compared to normal tissues, suggesting alterations in cellular redox status due to the enhanced expression of Trx might be asso-

ciated with the formation and development of carcinomas.[14] In colon cancer cell lines, TrxR mRNA is upregulated after treatment with bile acids that mimic oxidative stress.[15] TrxR, highly expressed in lung carcinomas, has been shown to take part in the activation of transcriptional factors and regulation of apoptosis in non-small cell lung carcinomas.[16] However, in high-grade tumors, the TrxR expression is diminished, suggesting loss of redox regulation in tumors with low differentiation.[16] In the livers of selenium-deficient rodents, inhibition of TrxR by a low dose of gold, in the form of aurothioglucose, leads to the induction of hepatic heme oxygenase-l activity, suggesting that loss of TrxR is responsible for the induction of heme oxygenase-l due to selenium deficiency.[17] The effects of anti-tumor drugs such as carmustine and cisplatin are partly explained by the inhibition of TrxR.[18] Consistently, high levels of TrxR can support drug resistance. Understanding the regulatory mechanism of TrxR expression in tumor cells will lead to further elucidation of tumor physiology and its application. Until now, the differences in the regulation of TrxR between tumor cells and their corresponding normal cells have not been clearly understood. Herein, we examined the effects of various stresses on the TrxR activities in human normal hepatic and hepatoma cell lines, Chang and HepG2, as a preliminary study to address whether the TrxR-mediated defense system against stresses is different between normal and tumor cells.

MATERIALS AND METHODS

Cell lines

The human normal liver cell line, Chang (American Type Culture Collection Number: CCL-13), and the human hepatocyte carcinoma cell line, HepG2, were kindly provided by Dr. K. W. Kim (Seoul National University, Korea). These cell lines were grown in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) heat-inactivated FBS (Biowhittaker Inc. Walkerville, MD, USA), 100 units/ml penicillin and 100 μg/ml streptomycin. Both cell lines were grown at 37℃ in a humidified air/CO_2 (19 : 1) atmosphere.

Treatment and cell harvest

To examine the effects of various stress-inducing reagents on TrxR activity, the normal hepatic Chang and the hepatoma HepG2 cells were cultured with 90% confluence. The cells were then treated with various metals, selenite, hydrogen peroxide, lipopolysaccharide (*Escherichia coli* serotype 026 : B6, Sigma-Aldrich Co., St. Louis, MD, USA), superoxide- or NO-producing agents at the concentrations shown in Table 1 for 2 or 3 h, followed by centrifugation at 1000 × g for 5 min.

Preparation of cytosolic extracts

Cytosolic extracts from the human cell lines were prepared in animal cell lysis buffer (50 mM HEPES, 10% sucrose, 0.1% Triton X-l00, pH 7.5) and centrifugation at 12,000 × g for 15 min. The protein concentrations were determined according to the method of Bradford, using bovine serum albumin as a standard.[19]

Enzymatic assay

The thioredoxin reductase activity was measured as the reduction of DTNB (Sigma-Aldrich Co., st. Louis, MD, USA) in the presence of NADPH (Sigma-Aldrich Co., St. Louis, MD, USA).[20] The assay mixture contained 0.2 M phosphate buffer (pH 7.6), 1 mM EDTA, 0.25 mM NADPH and 1 mM DTNB. The increase in the absorbance at 412 nm was monitored over 5 min at 25℃.

MTT reduction assay

The cell viability was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[21] Briefly, cells incubated with various stress inducing agents were treated with 5 μl of MTT (Sigma-Aldrich Co., St. Louis, MD, USA) solution (5 mg/ml) for 2 h. The cells were then lysed with isopropyl alcohol, and the absorbance read at 540 nm.

RESULTS AND DISCUSSION

The levels of expression or activity of defense enzymes may play important roles in the survival and growth of cancer cells. The thioredoxin system is a major line of cellular defense against oxygen damage. In this study, the regulation of thioredoxin reductase (TrxR) was investigated in the human normal hepatic cell line, Chang, and the human hepatoma cell line, HepG2. These two cell lines were chosen with the following assumption; although the Chang cell line is normal, while the HepG2 cell line is cancerous, their original phenotype must be the same, as both are derived from human liver cells. However, the biochemical differences between the Chang and HepG2 cell lines in response to stresses remain to be uncovered. Moreover, the regulation of TrxR has never been compared between the Chang and HepG2 cell lines. Thus, by comparing the TrxR regulatory patterns in the two cell lines, how tumorigenesis may influence the regulation of TrxR activity could be estimated.[22]

Cell viability

The viabilities of the Chang and HepG2 cells responding to the various agents used in this work were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[21] The other agents, with the exception of menadione, did not significantly affect the viabilities of the Chang and HepG2 cell lines (Table 1), but in the presence of menadione (1 mM), the viabilities of the two cell lines decreased to 50% and 64%, respectively (Table 1). Therefore, the TrxR induction effect of most of the agents examined in this study did not originated from a decreased viability.

Metals

Various metals have been thought to induce harmful oxidative stresses through lipid peroxidation, which increases the activity of various anti-oxidative enzymes, including TrxR, glutathione reductase and catalase.[4,23-26] To compare the effects of metals on TrxR activity between human

Table 1. Cytotoxic Effects on the Survivals of Human Normal Hepatic and Hepatoma Cell Lines of the Various Agents used in This Study

Agents	Concentration	Relative Survival[a]	
		Chang	HepG2
Control	-	100.0 ± 021.1	100.0 ± 8.8
Aluminum chloride	0.5 mM	110.3 ± 2.8	110.6 ± 2.8
	1.0 mM	115.3 ± 2.4	113.1 ± 8.5
Cadmium chloride	0.1 mM	110.6 ± 18.3	93.8 ± 6.4
Cupric chloride	0.2 mM	85.3 ± 11.9	80.9 ± 10.8
Zinc chloride	0.5 mM	90.8 ± 24.3	84.8 ± 18.0
Manganese chloride	2.0 mM	94.0 ± 17.9	84.8 ± 6.4
Sodium selenite	0.02 mM	132.1 ± 13.8	104.4 ± 9.0
Menadione	0.5 mM	92.7 ± 2.8	54.4 ± 7.7
	1.0 mM	50.0 ± 4.4	36.5 ± 10.8
Sodium nitroprusside	3.0 mM	104.6 ± 3.7	98.2 ± 3.6
	6.0 mM	77.1 ± 12.4	84.5 ± 24.0
H_2O_2	5.0 mM	112.3 ± 8.0	128.1 ± 9.1
Lipopolysaccharide	2 μg/mL	107.1 ± 19.1	98.2 ± 8.7
No serum	-	104.1 ± 6.0	88.9 ± 7.7

[a]Relative survival values, after the treatment of various reagents, were calculated by considering the survival of untreated cell lines (control) to be 100. The cell viability was measured as described in 'Materials and Methods'. Data represent the mean ± SD.

normal hepatic and human hepatoma cell lines, both the Chang and HepG2 cell lines were cultured with 90% confluence and treated with various metals for 3 h.

As shown in Fig. 1 and 2A, most of the metal treatments increased the TrxR activities in both the Chang and HepG2 cell lines. Although cupric chloride (0.2 mM) increased the TrxR activities by about 1.94- and 2.53-fold in the Chang and HepG2 cell lines, respectively, the degree of enhancement was slightly higher in the Chang cell line, or similar between the two cell lines. For example,

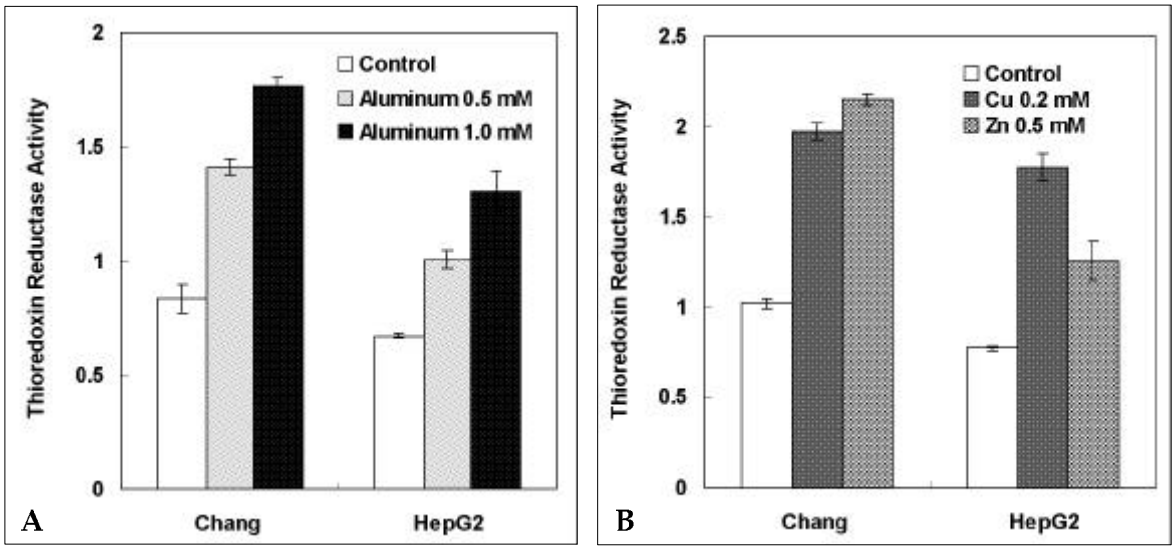

Fig. 1. The effects of various metal ions on the thioredoxin reductase activity in human normal hepatic and hepatoma cell lines. (A) aluminum chloride (0.5 and 1.0 mM) and (B) cupric chloride (0.2 mM) and zinc chloride (0.5 mM). The hepatic cells were grown and harvested 3 h after treatment, and the cell extracts prepared as described in 'Materials and Methods'. Experiments were performed in triplicate. Thioredoxin reductase activity means the specific enzyme activity (Δ412/min/μg protein). Data represent the mean ± SD.

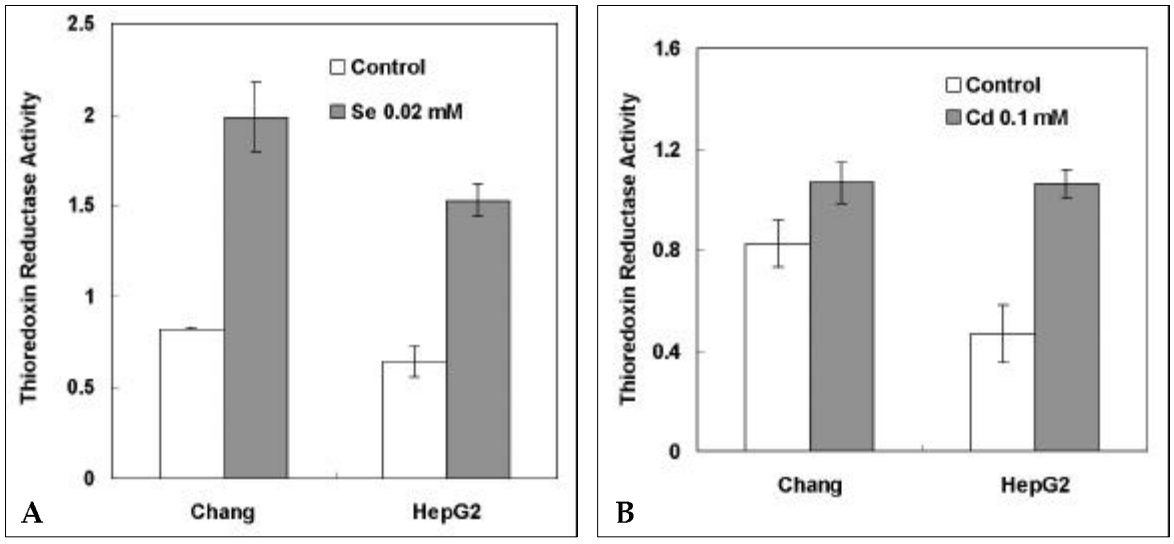

Fig. 2. Inducible effects of sodium selenite (0.02 mM) (A) and cadmium chloride (0.1 mM) (B) on the thioredoxin reductase activity in human normal hepatic and hepatoma cell lines. The hepatic cells were grown and harvested 3 h after treatment, and the cell extracts prepared as described in 'Materials and Methods. Experiments were performed in triplicate. Thioredoxin reductase activity means the specific enzyme activity (Δ412/min/μg protein). Data represent the mean ± SD.

aluminum chloride (1 mM), zinc chloride (0.5 mM) and sodium selenite (0.02 mM) elevated the TrxR activity by about 2.31-, 2.11- and 2.41-fold in the Chang cell line, and by 1.95-, 1.63- and 2.37-fold in the HepG2 cell line, respectively. As a constituent of TrxR, selenite appeared to be involved in the induction of TrxR in human hepatic cells, although there was not a significant difference in the enhancement between the Chang and HepG2 cell lines. Strong inhibitory effects, particularly on mammalian tumor cell growth, were observed at higher doses, in contrast to the stimulatory effects on both the normal and tumor cells found at low (nM range) concentrations.[27] Although the mechanisms are not clearly characterized, the known inhibitory effects of selenite include NADPH depletion, competitive inhibition of TrxR, and oxidation of catalytic and structural cysteines in human Trx.[28] The stimulatory effect of selenite at low doses may be explained by the facilitation of the synthesis of selenoproteins, like TrxR, which could be supported by our results.

The treatment with cadmium chloride (0.1 mM) induced the TrxR activities in both the Chang and HepG2 cell lines. However, the activities were enhanced about 1.30- and 2.28-fold in the Chang and HepG2 cell lines, respectively (Fig. 2B), showing a much higher TrxR activity in the HepG2 than in the Chang cell line following its induction by the treatment with cadmium chloride. Even though the heavy metal induction of mammalian TrxR has never been reported before, cadmium and zinc were shown to induce several morphological and biochemical effects related to salient features of programmed cell death. In C6 rat glioma cells, cadmium has been shown to cause externalization of phosphatidylserine, breakdown of the mitochondrial membrane potential, activation of caspase-9, internucleosomal DNA fragmentation, chromatin condensation and nuclear fragmentation.[29] Furthermore, great differences in cadmium-induced apoptosis were found with different cell lines. Rat C6 glioma cells turned out to be the most sensitive, with an IC_{50}-value of 0.7 μM, while human A549 adenocarcinoma cells were relatively resistant, with an IC_{50}-value of 164 μM. Despite the treatment with 100 μM cadmium chloride, this did not affect the viabilities of the HepG2 and Chang cell lines.[29] The increased TrxR may be responsible for this cytoprotective effect against the cytotoxicity of cadmium, via the direct inhibition of apoptosis signal-regulating kinase (ASK) 1 or activation of NF-κB, a transcription factor required for cell proliferation, as Trx R and thioredoxin regulate the activation of NF-κB synergistically.[30,31] Recently, a proteome analysis of the cadmium response in *Saccharomyces cerevisiae* identified 54 induced and 43 suppressed proteins.[32] The strong inductions included several proteins with antioxidant properties, such as Trx, TrxR and GSH. The expressions of some of these proteins are dependent on the AP1-like transcriptional factors, Yap1 and Skn 7, which regulate the adaptive response to oxidative stress[33,34]. Therefore, it is possible that different sensitivities to various metals between normal and cancer cell lines influence the expressions of a number of proteins, which ultimately lead to the different degrees of TrxR induction.

Taken together, the TrxR activity has been shown to be regulated by various metal ions. The degree of enhancement varies between Chang and HepG2 cell lines, but largely depends on the metal species and concentrations.

Menadione, hydrogen peroxide, and lipopolysaccharide

Menadione (2-methyl-1,4-naphthoquinone) is a quinone-containing substance widely used as an experimental tool for the induction of oxidative stress in cells and tissues. Menadione (MD) can undergo a one-electron reduction to a semiquinone radical, which in the presence of O_2 can be reoxidized to the parent compound, with the concomitant formation of a superoxide anion (O_2).[35] Menadione treatment (0.5 and 1 mM) markedly elevated the TrxR activities by about 1.82- and 1.85-fold, and 1.56-and 2.09-fold in the Chang and HepG2 cell lines, respectively (Table 2).

Hydrogen peroxide is a central oxygen metabolite produced in several cellular compartments and the source of other reactive oxygen species, e.g., highly reactive hydroxyl radical.[36] Hydrogen peroxide (5 mM) produced a significant increase in the TrxR activity of the Chang and HepG2 cell lines. Its activity was increased 1.26- and 4.60-fold in the Chang and HepG2 cell lines, respectively,

Table 2. Effects of Menadione, Hydrogen Peroxide and Sodium Nitroprusside on the Thioredoxin Reductase Activity in Human Normal Hepatic and Hepatoma Cell Lines

Agents	Cell lines	Relative TrxR activity[a]
Menadione		
(0.5 mM)	Chang	1.82 ± 0.15
	HepG2	1.56 ± 0.05
(1.0 mM)	Chang	1.85 ± 0.20
	HepG2	2.09 ± 0.05
Hydrogen peroxide		
(5.0 mM)	Chang	1.26 ± 0.06
	HepG2	4.60 ± 0.37
Sodium nitroprusside		
(3.0 mM)	Chang	1.34 ± 0.15
	HepG2	1.67 ± 0.05
(6.0 mM)	Chang	2.72 ± 0.40
	HepG2	2.91 ± 0.01

[a]Relative thioredoxin reductase activity was calculated by considering the specific activity of corresponding untreated cells to be 1. After 2h-treatment, TrxR assays were performed in triplicate, as described in 'Materials and Methods'. Data represent the mean ± SD.

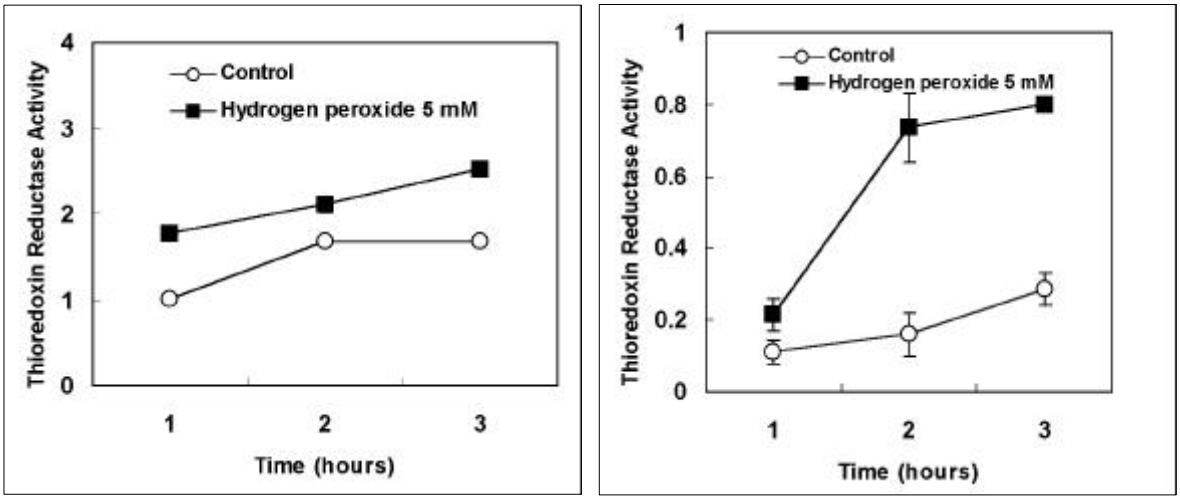

Fig. 3. Time-dependent inducing effect of hydrogen peroxide (5 mM) on thioredoxin reductase activity in Chang (A) and HepG2 (B) cell lines. The hepatic cells were grown and harvested 1, 2 and 3 h after treatment, and the cell extracts prepared as described in 'Materials and Methods'. Experiments were performed in triplicate. Thioredoxin reductase activity means the specific enzyme activity (Δ412/min/μg protein). Data represent the mean ± SD.

2 h after treatment (Table 2). Hydrogen peroxide treatment brought about a much higher TrxR induction in the HepG2 cell line at all given incubation times (Fig. 3). Both the TrxR mRNA and protein levels have previously been shown to be markedly increased by treatment of the peroxynitrite donor, SIN-1, in human umbilical vein endothelial cells (HUVEC).[37] Together with our finding, this implies that mammalian TrxR is subject to regulation by reactive oxygen and nitrogen species. However, the higher TrxR induction in the HepG2 cell line by hydrogen peroxide suggests the involvement of other oxidative stress factors. The induced TrxR may sequentially regulate the AP-1 activity via a cysteine motif located in the protein.[38]

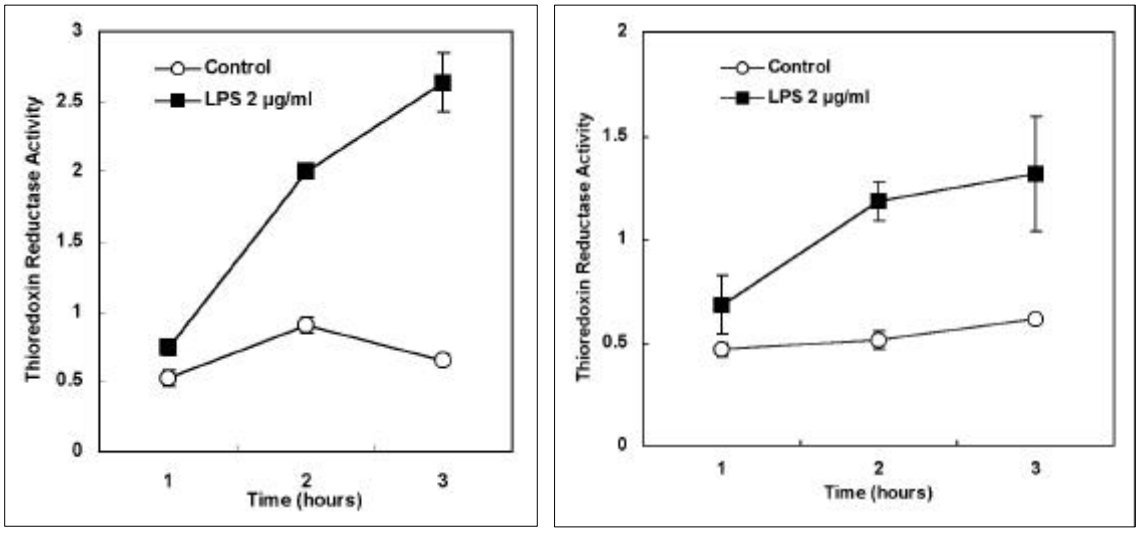

Fig. 4. Time-dependent inducing effect of lipopolysaccharide (2 μg) on the thioredoxin reductase activity in Chang (A) and HepG2 (B) cell lines. The hepatic cells were grown and harvested 1, 2 and 3 h after treatment, and the cell extracts prepared as described in 'Materials and Methods.' Experiments were performed in triplicate. Thioredoxin reductase activity means the specific enzyme activity (Δ412/min/μg protein). Data represent the mean ± SD.

Lipopolysaccharide (LPS) induces a variety of cytokines, such as TNF-α, interleukin-l0 (IL-10), IL-6 and IL-8, and is also involved in producing ROS.[39,40] The treatment with LPS (2μg/mL) largely induced TrxR activity in the Chang and HepG2 cell lines in time-dependent manners (Fig. 4). After a 3 h-treatment with LPS, the TrxR activities were elevated to about 4.02- and 2.2-fold in the Chang and HepG2 cell lines, respectively, implying that the induction of TrxR is associated with the oxidative stressful effect of LPS. Additionally, although the LPS-mediated signaling pathway is not completely understood, LPS is involved in the activation of the NF-κB and MAP kinase pathways through its receptor, TLR-4 (toll-like receptor)/MD-2, indicating a possible role of the TrxR system in the LPS-signaling pathway.[41] Moreover, the LPS-mediated-TrxR induction is consistent with the previous report that LPS approximately quadruples the TrxR expression in the placentae of pregnant mice.[42] Collectively, oxidative stress is involved in the regulation of the TrxR activity in human liver cells.

Nitric oxide

Nitric oxide (NO), secreted from various cell types, contributes to a variety of regulatory processes, such as neurotransmission, vasodilation and platelet aggregation.[43] In addition to these physiological effects, all of which are mediated via the activation of soluble guanylate cyclase, NO is also known to have bactericidal and tumoricidal potentials.[44] Released in large amounts in response to cytokines and/or endotoxins, NO participates in the cytotoxic activities of macrophages.[45] Diverse targets on the cell surface, as well as within the cell, including the thiol groups of proteins, have been suggested to account for the cytotoxicity of NO. NO is thought to inhibit key enzymes in mitochondrial respiration, DNA synthesis and iron metabolism.[43]

NO-generating sodium nitroprusside (3 and 6 mM) induced significantly enhanced the TrxR activities in the Chang and HepG2 cell lines (Table 2) by 1.34- and 2.72-fold, and 1.69- and 2.91-fold in the Chang and HepG2 cell lines, respectively (Table 2). Its induction effect at both 3 and 6 mM in the HepG2 cell line was higher than in the Chang cell line. The TrxR activity in human hepatic cells is induced by nitrosative stress. Our result corresponds with the enhanced TrxR mRNA level caused by reactive nitrogen species.[37]

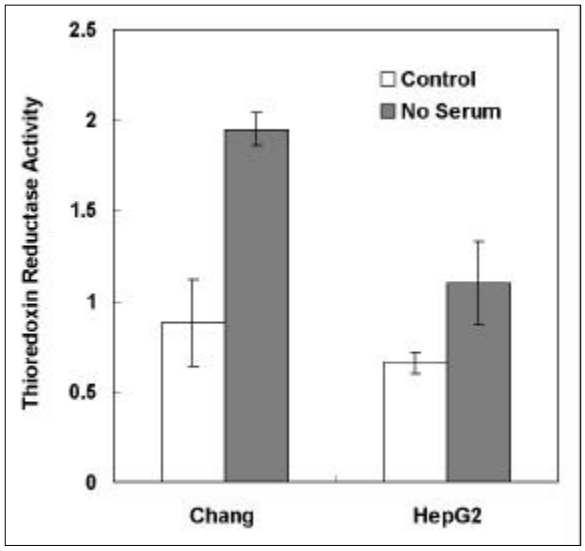

Fig. 5. Effect of the absence of fetal bovine serum on the thioredoxin reductase activity in human normal hepatic and hepatoma cell lines. The hepatic cells were grown in DMEM plus 10% FBS, and then cultured in the serum-free DMEM for 3 h. The cell extracts for the enzyme assays were prepared as described in 'Materials and Methods'. Experiments were performed in triplicate. Thioredoxin reductase activity means the specific enzyme activity (Δ412/min/μg protein). Data represent the mean $\pm$ SD.

Lack of serum

Andoh et al. suggested that serum deprivation causes an increase in both nitric oxide and · OH generation in the human neuroblastoma cells, SH-SY2Y.[46,47] For serum deprivation, the DMEM media containing 10% FBS was changed to serum-free DMEM, with the two cell lines cultured in under serum-free conditions for 3 h. Fig. 5 shows that the TrxR activities in the Chang and HepG2 cell lines were enhanced by 2.23- and 1.67-fold, respectively, in the absence of fetal bovine serum. In the HepG2 cell line, a lower enhancement was observed than in the Chang cell line in the absence of serum. Enhancement of the TrxR activity due to serum deprivation might be mediated via increased levels of nitric oxide and oxidative stress.[46-48]

In conclusion, we have demonstrated that TrxR, a redox active enzyme implicated in the control of cell proliferation and transformation and also in the stress response, is induced by heavy metals, oxidative and nitrosative stresses, and serum deficiency in both Chang and HepG2 cell lines. The TrxR induction studied in this work, appears to be independent of cell death, and its degree of induction is not the same between Chang and HepG2 cell lines. Especially, hydrogen peroxide is able to induce TrxR in the HepG2 cell line to a much higher degree. Our results suggest that the stress responses are different between normal and tumor cells, which might be linked with the physiology and survival of tumor cells. Furthermore, future studies should focus on the physiological mechanism that causes these different responses to various stresses between hepatic normal and tumor cell lines.

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