Loss of activity of the selenoenzyme thioredoxin reductase causes induction of hepatic heme oxygenase-1

Volker Mostert, Kristina E. Hill, Raymond F. Burk*

Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville, TN 37232-2279, USA

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Abstract The stress response enzyme heme oxygenase (HO)-1 is induced in livers of selenium-deficient rodents, probably to compensate for loss of certain selenoproteins. We sought to identify those selenoproteins. Selenium-replete mice with genetic deletion of selenoprotein P or glutathione peroxidase-1 did not have elevated hepatic HO activity, thus ruling out involvement of those selenoproteins in HO-1 induction by selenium deficiency. However, inhibition of thioredoxin reductase (TrxR) by a low dose of gold in the form of aurothioglucose led to induction of hepatic HO activity. Moreover, further induction by phenobarbital was observed. This HO-1 induction pattern is also seen in selenium-deficient mice. In the rat hepatoma cell line H4IIE, inhibition of TrxR by aurothioglucose or by 1-chloro-2,4-dinitrobenzene led to induction of HO-1. We conclude that loss of TrxR is responsible for the induction of HO-1 by selenium deficiency.

Key words: Selenium; Liver; Heme oxygenase; Thioredoxin reductase

1. Introduction

Dietary selenium deficiency causes a decrease in selenoproteins, some of which have oxidant defense functions. This loss of antioxidant activity in selenium deficiency is accompanied by increased activities of several stress response enzymes that are not selenium-dependent. NAD(P)H:quinone reductase, glutathione *S*-transferase, γ-glutamyl-cysteine synthetase, and heme oxygenase (HO)-1 are two- to three-fold up-regulated in selenium-deficient rodent livers compared with selenium-replete controls [1–5]. These up-regulated enzymes have been associated with defense against oxidative stress.

In an earlier study we showed that HO-1 is expressed in selenium-deficient hepatocytes but not in control hepatocytes [5]. Moreover, the housekeeping heme oxygenase isoform HO-2 was unchanged in selenium-deficient hepatocytes. We reasoned that the activity of the induced HO-1 was likely to be compensating for an antioxidant function of a selenoprotein that was decreased in the selenium-deficient liver. In order to identify the selenoenzyme for which HO-1 compensates, we carried out experiments in which individual selenoenzymes were deleted or inhibited.

2. Materials and methods

2.1. Materials

All chemicals and enzymes were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Animals and treatments

Male weanling C57BL/6 mice were fed either a control or a selenium-deficient diet for 6 weeks following weaning (see [6] for diet composition). In experiments with aurothioglucose (ATG), the compound was dissolved in phosphate-buffered saline (PBS, 2.5, 5, 10, 20 mg/ml) and administered i.p. (1 ml/100 g body weight). In experiments with phenobarbital (PB), the compound was dissolved in 0.15 M NaCl (8 mg/ml) and administered i.p. (1 ml/100 g body weight) 6 h before the animal was killed. *GSHPx-1*$^{-/-}$ mice [7] were kindly provided by Dr. Y.S. Ho (Wayne State University, Detroit, MI, USA). Generation and characteristics of *Sepp*$^{-/-}$ (selenoprotein P knockout) mice are described elsewhere [8]. Mice were killed by exsanguination via the vena cava inferior under isoflurane anesthesia.

Livers were excised and homogenized in ice-cold 0.25 M sucrose, 100 mM potassium phosphate (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of each leupeptin and aprotinin. The homogenate was centrifuged at 18 000×*g* for 10 min at 4°C and the supernatant was used for HO assay.

2.3. Cell culture and treatment

The rat hepatoma cell line H4IIE was cultured in Dulbecco's modified Eagle's medium containing 2.5 g glucose per liter, non-essential amino acids, penicillin-streptomycin, L-glutamine, 10% horse serum, and 2.5% fetal calf serum. Cells were maintained at 37°C under 5% CO_2. ATG was dissolved in PBS, 1-chloro-2,4-dinitrobenzene (CDNB) was dissolved in ethanol. Cells were treated for the indicated length of time with graded amounts of thioredoxin reductase (TrxR) inhibitors or an appropriate volume of the respective vehicle.

2.4. Assays

HO activity was measured spectrophotometrically in 18 000×*g* supernatants as described earlier [9]. Protein was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Cytosolic glutathione peroxidase (GSHPx), phospholipid hydroperoxide glutathione peroxidase (PH-GSHPx), glutathione reductase (GR) activities, and cellular glutathione (GSH) levels were determined as previously described [3,10–12]. TrxR activities were determined essentially as described earlier [13].

2.5. Western blotting

Western detection of HO-1 protein was performed as described elsewhere [5]. Polyclonal HO-1 antibodies were a kind gift from Dr. C.D. Ferris (Vanderbilt University, Nashville, TN, USA).

2.6. Statistical analysis

Student's *t*-test was performed using Microsoft Excel 98 (Microsoft, Redmond, WA, USA).

*Corresponding author. Fax: (1)-615-343 6229.
E-mail address: raymond.burk@vanderbilt.edu (R.F. Burk).

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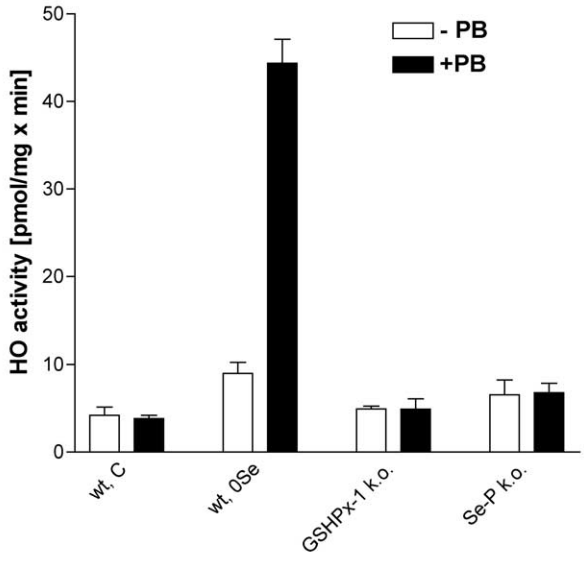

Fig. 1. HO activities in 18 000×*g* liver supernatants from mice that were untreated (empty bars) or treated with PB for 6 h (black bars). Unless indicated (0Se), mice were fed a control diet containing 0.25 ppm selenium as sodium selenite. Mice were either wild-type (wt) or homozygous gene knockout (k.o.) for the protein indicated. Bars indicate means ± S.E.M., $n = 3$–4.

3. Results

3.1. Hepatic HO activity in selenoprotein deficiency

Fig. 1 compares the hepatic HO activities of control and selenium-deficient mice with those of mice with specific selenoproteins deleted. As observed in selenium-deficient rats [14], selenium-deficient mice display hepatic HO activities twice as high as selenium-replete control mice; PB treatment caused an additional five-fold induction. In mice lacking either GSHPx-1 or selenoprotein P, hepatic HO activity was not elevated and was not influenced by PB treatment. Thus, neither GSHPx-1 loss nor selenoprotein P loss is associated with HO-1 induction.

3.2. ATG inhibits TrxR and induces HO

Mice homozygous for disrupted selenoprotein P or GSHPx-1 genes are the only animals with selenoprotein genes disrupted that have been reported viable thus far. In order to study the selenoenzyme TrxR, we resorted to inhibition of TrxR with ATG. ATG inhibits both GSHPx and TrxR. However, its IC_{50} for GSHPx is three orders of magnitude greater than for TrxR [15], indicating that TrxR can be inhibited with a dose of ATG that would not inhibit GSHPx. Eight hours following injection of mice with 100 mg ATG/kg, hepatic cytosolic GSHPx and PH-GSHPx activities were not affected, while cytosolic TrxR activity was depressed to 3% of untreated control values (Fig. 2). GR, an enzyme structurally and functionally related to TrxR, was also not inhibited by this ATG dose.

Table 1
HO activities in 18 000×*g* liver supernatants from mice that were fed diets containing the indicated amounts of selenium and treated with PB (80 mg/kg b.w., i.p.) where indicated

Se (ppm)	ATG (mg/kg b.w.)	HO (pmol/mg/min)[a]	
		−PB	+PB
0.25	0	4.2 ± 1.0[a]	3.8 ± 0.4[a]
0	0	9.0 ± 1.3[b]	44.4 ± 2.8[c]
0.25	100	13.2 ± 1.9[b]	41.9 ± 9.6[c]

[a]Values are means ± S.E.M., $n = 3$–4. Values not sharing a common superscript are significantly different from each other (Student's *t*-test, $P < 0.05$).

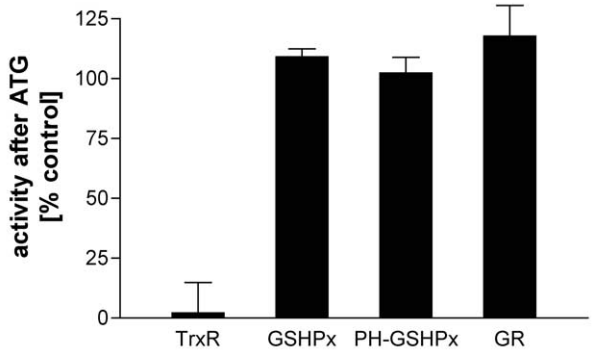

Fig. 2. TrxR, GSHPx, PH-GSHPx and GR activities in 18 000×*g* liver supernatants from mice that were treated with ATG (100 mg/kg b.w., 8 h, black bars) or left untreated (open bars). Bars indicate means ± S.E.M., $n = 3$.

The ATG treatment tripled HO activity in liver compared to HO activity in livers of untreated mice (Table 1). Treatment with PB 2 h after ATG treatment led to an additional three-fold elevation in hepatic HO activity in ATG-treated mice. Western analysis confirmed that both selenium deficiency and ATG treatment induced the expression of the HO-1 isoform in the liver and enabled its further inducibility by PB (Fig. 3). HO-1 had no appreciable expression in livers of selenium-replete control mice that had not received ATG. TrxR in murine liver exists in cytosolic (TrxR-1) and mitochondrial (TrxR-3) isoforms, although the former appears to be more abundant than the latter [16]. Under the conditions of the ATG experiment, TrxR activities in cytosolic and mitochondrial fractions of mouse livers were equally inhibited (data not shown).

3.3. Induction of HO-1 in rat hepatoma cells by ATG treatment

When rat hepatoma cells of the line H4IIE were treated with graded amounts of ATG for 16 h, an IC_{50} of approximately 25 μg ATG/ml was observed for TrxR activity (Fig. 4). HO-1 expression level increased substantially when TrxR activity fell below 4% of control activity. When ATG concentration was raised above 1000 μg/ml of medium, TrxR activity became undetectable, but HO-1 protein continued to increase, indicating that an induction mechanism independent of TrxR inhibition might function at higher concentrations of ATG.

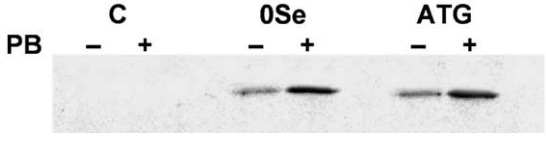

Fig. 3. Representative Western detection of HO-1 in 18 000×*g* liver supernatants from mice that were untreated or treated with PB for 6 h. C and ATG mice were fed a control diet containing 0.25 ppm selenium as sodium selenite. 0Se mice were selenium-deficient. The ATG mice received ATG (100 mg/kg, i.p.) 8 h before being killed. Where indicated, PB was given 2 h after ATG administration.

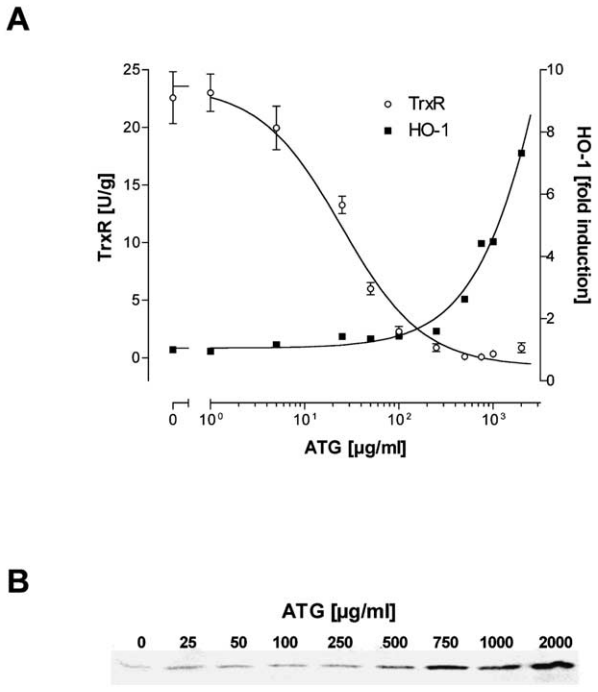

Fig. 4. TrxR activity (open circles) and HO-1 protein expression (solid squares) in 18 000×*g* supernatants from H4IIE cells. Cells were treated with the indicated doses of ATG for 16 h. TrxR activities are given as means ($n = 3$, error bars indicate S.E.M.). HO-1 expressions were determined by densitometric evaluation of the respective Western blots. Symbols indicate means of two independent experiments (A). A representative blot is shown in B.

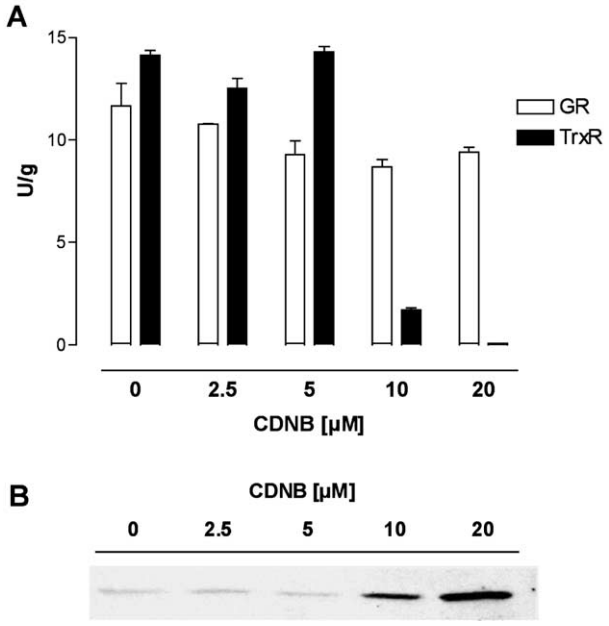

Fig. 5. TrxR and GR activities (A) and HO-1 protein expression (B) measured in 18 000×*g* supernatants from H4IIE cells that had been treated with the indicated doses of CDNB for 4 h. Enzyme activities are given as means ($n = 3$, error bars indicate S.E.M.).

3.4. Induction of HO-1 after inhibition of TrxR by CDNB

We investigated whether CDNB, an inhibitor of TrxR that is chemically unrelated to ATG, can elicit HO-1 induction in cultured liver cells. CDNB alkylates TrxR and irreversibly inhibits it [17,18]. CDNB is also conjugated with GSH by various glutathione *S*-transferase isoenzymes and depletes GSH through this mechanism [19]. As shown in Fig. 5, CDNB caused a dose-dependent inhibition of cytosolic TrxR accompanied by an increase of HO-1 protein expression. GR activities were not significantly reduced. Like ATG, CDNB inhibited TrxR activities in both cytosolic and mitochondrial fractions of H4IIE lysates (not shown).

CDNB depletes cellular GSH and CDNB-treated H4IIE cells have low GSH levels. To eliminate GSH depletion as the cause of HO-1 induction [20], we added graded amounts of the GSH precursor, *N*-acetylcysteine (NAC), to the culture medium to maintain normal GSH concentrations in CDNB-treated cells. As shown in Fig. 6, CDNB inhibited TrxR and induced HO-1 even when control GSH levels were maintained by NAC supplementation. This suggests that CDNB treatment leads to HO-1 induction through a mechanism unrelated to GSH depletion. TrxR inhibition is likely to be that mechanism.

4. Discussion

Earlier work from our group demonstrated that hepatic HO activity is increased in selenium-deficient rats and is further induced by PB treatment of them [14]. Injection of deficient animals with selenium 6 h before PB treatment blocked the HO induction, whereas its administration simultaneously with PB had no effect on induction [4]. This suggested that selenium administration was supporting synthesis of a selenoprotein that prevented HO induction. The aim of the present study was to identify that selenoprotein.

Two selenoproteins, GSHPx-1 and selenoprotein P, were easily eliminated by the use of animals in which genes for these proteins had been disrupted. Another approach had to be taken for TrxR because no mice with a TrxR gene deleted are available. Attempts to produce mice with the Trx gene deleted were unsuccessful because the deletion was embryonic lethal [21]. Thus, production of mice with TrxR genes deleted may only be possible with tissue-specific deletion techniques.

TrxR is exquisitely sensitive to inhibition by gold compounds [22]. We were able to inhibit it in mouse liver without inhibiting GSHPx-1 (GSHPx activity) or GSHPx-4 (PH-

Table 2
Overview of antioxidant enzymes inducible by selenium deficiency or gold compounds

Enzyme	Induced by	
	Selenium deficiency	Gold compounds
γ-Glutamyl-cysteine synthetase	enzyme activity [3]	mRNA [28]
NAD(P)H:quinone reductase	enzyme activity [1], mRNA [29]	mRNA [28]
Glutathione *S*-transferase	enzyme activity [2]	enzyme activity [30,31]
HO-1	enzyme activity [4], protein expression [5], mRNA [5]	enzyme activity [this study], protein expression [this study], mRNA [28]

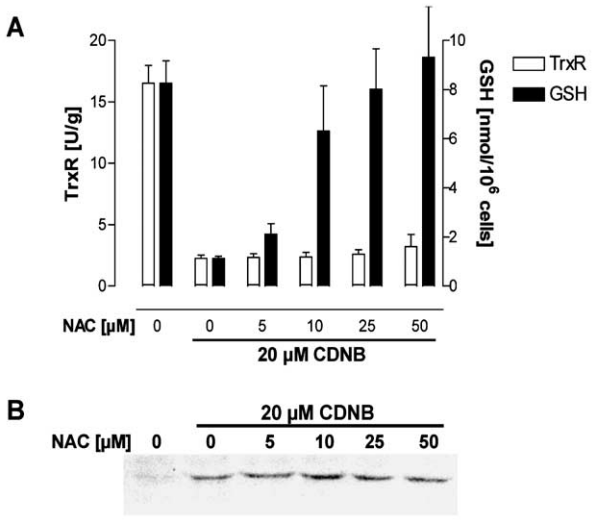

Fig. 6. TrxR activities and GSH levels in 18 000×*g* supernatants from H4IIE cells (A). Cells were treated with 20 μM of CDNB for 4 h in the presence of the indicated concentrations of NAC. Values are given as means ($n=3$, error bars indicate S.E.M.). Representative HO-1 protein expression is shown in B.

GSHPx activity) by administering ATG (Fig. 2). Inhibition of hepatic TrxR in vivo was associated with induction of HO-1 and with further inducibility of HO-1 by PB (Fig. 3). This behavior of HO-1 is the same as seen in selenium deficiency. Work in the rat hepatoma cell line gave the same effect with ATG as seen in vivo and demonstrated that CDNB, another TrxR inhibitor, caused HO-1 induction as well. This strongly suggests that lack of TrxR, or of an activity dependent on it, leads to the induction of HO-1.

The thioredoxin enzyme family consists of the TrxRs, the Trxs, and the enzymes that derive their reducing equivalents from TrxR through Trx [23]. Most peroxiredoxins fall into this category. Another of these enzymes, methionine sulfoxide reductase B, is a selenoprotein [24], and therefore doubly dependent on selenium. Thus, inhibition of TrxR affects many enzyme activities. One (or more than one) of these TrxR-dependent enzymes is likely to be the enzyme for which HO-1 compensates.

Besides its antioxidant function, the thioredoxin family fulfills a crucial role in the regulation of redox-sensitive transcription factors, e.g. the DNA-binding activity of AP-1 is regulated by Trx [25,26] and especially the AP-1-dependent induction of HO-1 by inflammatory mediators is facilitated by Trx [27]. In a previous study, we found increased AP-1 binding activity preceding HO-1 induction in selenium-deficient rat liver in response to PB [5]. This indicates that Trx-dependent gene regulation might be the molecular basis for HO-1 induction by selenium deficiency and thus TrxR deficiency. Several antioxidant enzymes are induced in selenium deficiency. At least four of them are also inducible by antirheumatic gold compounds which function as TrxR inhibitors (Table 2). It is therefore conceivable that TrxR is the key selenoprotein associated with the induction of these stress response genes by dietary selenium deficiency.

While the Trx family has been identified as having a special relationship with HO-1, further work will be necessary to identify the family members that are directly involved.

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