Whole-body X-irradiation Induces Acute and Transient Expression of Heme Oxygenase-1 in Rat Liver

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Activation of the stress-inducible heme oxygenase-1 (HO-1) gene by X-irradiation was investigated in rat liver. When male Wistar MS strain rats (8 weeks) received whole-body irradiation of 17.0 Gy, 7 h later the activity of heme oxygenase in the liver was significantly enhanced (2.5 times). The level of HO-1 mRNA expression was increased by 2.3 and 4.0 times 2 and 4 h after radiation, and then declined at 7 and 10 h to the level of 2.0 and 1.6 times of the control. When the X-ray dose was varied from 4.0 to 21.7 Gy, the transcription of the gene was enhanced at all doses and the level of activation was dose-dependent. Finally, western blotting of irradiated liver demonstrated a significant increase in the level of HO-1 induced by X-rays, peaking at 4 h. Thus, X-rays were confirmed to be stressors that induce acute HO-1 expression transiently in the liver.

INTRODUCTION

The transcription of the heme oxygenase-1 (HO-1) gene is enhanced by various stimuli, such as oxidative stress, UVA radiation and heat shock, and a considerable body of evidence has confirmed that the HO-1 gene is cytoprotective against numerous stresses, as reviewed by Maines1). It is also commonly called Hsp (heat shock protein) 32. Biliverdin, which is one of the products of an enzyme reaction by heme oxygenase, is subsequently converted to bilirubin by biliverdin reductase in mammals. Biliverdin and bilirubin are powerful antioxidants, and it was reported that a pretreatment with bilirubin prevented UVA-induced injury in rats2). Another product of the enzyme reaction by heme oxygenase is carbon monoxide, which is considered to be a promising and significant messenger molecule in the soluble guanylate cyclase (sGC)-cGMP system1).

Ionizing radiation produces reactive oxygen species (ROS), as detected by an increased decay rate of a spin probe3), and ROS exerts diverse biological effects on cells and animals4,5). The induction of HO-1 by ionizing radiation was reported in rat liver at 5 to 7 days6) and in rat kidney at 50 to 65 days7) postirradiation. However, an immediate early effect of radiation upon the HO-1 gene had not been characterized. In this report, we provide evidence that the heme oxygenase-1 gene is induced by ionizing radiation in rat liver within a few hours.

MATERIALS AND METHODS

The activity of heme oxygenase was measured based on a method of Yoshida and Kikuchi8) in the
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presence of hemin, NADPH, rabbit liver NADPH-cytochrome P450 reductase (Sigma-Aldrich) and a partially purified biliverdin reductase preparation from rat liver. Male Wistar MS strain rats at 2 months of age (Japan SLC) received whole-body irradiation with X-rays (dose rate, 0.70 Gy/min.), and were euthanatized with CO₂ suffocation times after irradiation, as indicated. The livers were dissected out and processed without perfusion with saline to remove blood, because the activity of heme oxygenase was not affected, even though the liver was perfused before preparing the microsomal fraction. The liver was homogenized in 0.25 M sucrose-50 mM Tris-HCl buffer (pH 7.4), and the microsomal fraction was prepared. A partially purified fraction of biliverdin reductase was prepared from the cytosol fraction of the liver from a normal male rat according to a method of Tenhunen et al 9. Biliverdin produced by heme oxygenase was converted to bilirubin by a sufficient amount of biliverdin reductase, and finally bilirubin was measured photometrically.

Probes for northern blot analyses were obtained as follows. Primers used for the amplification of rat HO-1 cDNA, 5’-GACATGGCCTTCTGGTATGG-3’ for sense and 5’-TCATGCGAGCACGATAG GC-3’ for antisense, were designed so as not to contain homology sequences with HO-2 or HO-3 10-12. A cDNA library was obtained from the total RNA of rat ovary using a First-strand cDNA Synthesis Kit (Amer sham Pharmacia Biotech), and a 286-bp fragment of HO-1 cDNA was amplified by a PCR reaction from the library. A fragment was subcloned into a cloning vector, pCR2.1-TOPO (Invitrogen). The obtained fragment of 286-bp rat HO-1 cDNA was confirmed by sequencing. A 803-bp cDNA probe of murine glycer aldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control in northern blotting. It was amplified by a PCR reaction from the same rat-ovary cDNA library as the template. Primers used for the GAPDH PCR were 5’-AAACCATCACCATCTTC TTCA-3’ for sense and 5’-CAGGGTTTCTTTACCTCTG-3’ for antisense 13, and the sequence of the GAPDH PCR product was confirmed. An mRNA fraction was prepared from liver with a QuickPrep Micro mRNA Purification Kit (Amer sham Pharmacia Biotech), and separated by electrophoresis on a 1% agarose/2.2 M formaldehyde denaturing gel in MOPS buffer. This was blotted onto a Gene Screen Plus nylon membrane (NEN). The radiolabeled probe was generated by random priming of DNA fragments with Ready-to-Go DNA Labeling Beads (Amer sham Pharmacia Biotech) using α-32P-dCTP (AA0075, Amer sham Pharmacia Biotech). Membranes were hybridized at 42°C with a radiolabeled rat HO-1 probe (5×SSC, 1% SDS). Blots were then washed at 50°C (2×SSC) and HO-1 hybridization signals were detected and quantified using a Bio imaging Analyzer System 2000 (Fuji Film). The size of the mRNA of HO-1 gene was confirmed to be 1.8 kb, as reported by Raju and Maines 14. After signal quantitation, the probes were stripped by boiling in distilled water for 10 min 15, the membrane was then re-hybridized with a 32P-labeled GAPDH probe as a loading control. The size of the mRNA of the GAPDH gene was confirmed to be 1.4 kb, as reported by Calvo et al 16. The amount of HO-1 mRNA was normalized against GAPDH mRNA expression.

To investigate the level of HO-1 protein in X-ray exposed rat liver, 20 µg of the 2000×g supernatant of the liver homogenate was subjected to SDS-PAGE under denaturing conditions, and transferred to a PVDF membrane (Bio-Rad). HO-1 protein was visualized by using a mouse monoclonal antibody against rat HO-1, diluted 1:280 (OSA-111, StressGen), with alkaline phosphatase-conjugated goat anti-mouse IgG, diluted 1:5000 (Cappel) as the secondary antibody, and visualized using Western Blot Chemiluminescence Reagent NEL100 (NEN). Detection and quantification were made using a chemiluminescence imaging system, LAS 1000 and Image Gauge Software (Fuji Film).

The effect of irradiation on HO-1 mRNA was analyzed by a one-factor analysis of the variance (ANOVA). For a comparison between the two mean values, the level of significance of the difference was evaluated by a t-test.
RESULTS AND DISCUSSION

We first measured the activity of heme oxygenase in the liver 7 h after irradiation with 17 Gy X-rays, because it had been reported that UVA irradiation to rat skin enhanced the heme oxygenase activity in the liver 6 h later\(^2\). As a result, the activity in the microsomal faction of irradiated rat liver was significantly higher (2.5 times) than the control value (Fig. 1). Thus, the activity of a stress-inducible enzyme, HO-1, was acutely enhanced in the liver by ionizing radiation.

In the next step, HO-1 mRNA expression was examined by northern blotting to investigate the transcriptional activation of this gene. Rats were irradiated with X-rays (17 Gy), and the mRNA fraction was prepared from the liver at 2, 4, 7 and 10 h after radiation. 6 to 7 µg mRNA preparations were separated by electrophoresis and blotted onto a nylon membrane, which was hybridized with a \(^{32}\)P-labeled rat HO-1 probe. As shown in Figs. 2A and 2B, the intensity of the signal increased significantly 2 h after irradiation (2.3 times), reached the maximal value (4.0 times) at 4 h, and then declined at 7 h (2.0 times) and 10 h (1.6 times). Thus,

**Fig. 1.** Effect of X-irradiation upon the activity of heme oxygenase in the liver. The microsomal fraction was obtained from each rat liver 7 h after irradiation. Mean±SE, three rats per group. Brackets enclose SE. *Significantly different from the control, P<0.02.

**Fig. 2A.** Transient upregulation of rat HO-1 mRNA after 17 Gy X-ray irradiation. The upper panel indicates the mRNA expression of the rat HO-1 gene. The lower panel indicates the expression of rat GAPDH mRNA as a loading control for northern blotting. The numbers above each lane indicate hours after X-ray irradiation (IR). In the leftmost lane (0), the mRNA sample was extracted from intact rat liver and 6 to 7 µg of mRNA was loaded as a non-irradiated control of HO-1 mRNA expression. In the lower panel, the Gene Screen Plus membrane used in the northern hybridization for HO-1 probe (upper panel) was boiled to remove the HO-1 probe and rehybridized with the murine GAPDH probe as a loading control. Details in Materials and Methods.

**Fig. 2B.** Change of HO-1 mRNA. Mean±SE, 4 (0, 7 and 10 h), 3 (2 h) or 6 (4 h), animals per group. a, P<0.01 and b, P<0.02, both significant differences versus control.
the elevation of the activity of heme oxygenase was confirmed to be due to an increase in HO-1 gene mRNA expression.

To confirm that the above-mentioned induction of HO-1 gene in rat liver is radiation-dependent, rats were irradiated with X-rays of 3.8, 7.6, 12.3, 17.0 and 21.7 Gy, and the level of HO-1 mRNA expression was examined by northern blotting. As a result, HO-1 mRNA expression was enhanced at each increased X-ray dose, and the extent of the enhancement was almost linear (Fig. 3). Thus, the induction of the HO-1 gene was confirmed to be a stress-response to radiation.

We then assessed the liver HO-1 protein level by a western blot analysis to determine if the transcriptional increase was reflected in protein expression. 20 µg protein from rat liver cytoplasm was subjected to SDS-PAGE under denaturing conditions on a 15% gel, transferred to a PVDF membrane and subjected to a western blot analysis. As a result, the HO-1 protein (32 kDa) level was observed to increase 2 h after irradiation (Fig. 4, upper panel), and reached a peak at 4 h. The increased level was sustained up to 10 h, though it had a tendency to decrease slightly. This experiment was performed in duplicate with similar results (data not shown). As a positive control signal for the irradiated liver, p53 was also blotted using the same prepa-

![Graph of Fold increase of HO-1 mRNA vs X-ray dose (Gy)](image)

**Fig. 3.** Dose-responsive induction of the HO-1 gene by X-rays. Messenger RNAs were obtained from rat liver 4 h after irradiation with various X-ray doses, and were subjected to northern blotting. The HO-1 mRNA signal was measured. *, P<0.001 and **, P<0.02.

![Image of western blot analysis](image)

**Fig. 4.** Change in HO-1 protein expression in the liver after X-irradiation detected by western blot analysis. The numbers above each lane indicate hours after X-ray irradiation (IR). Upper panel: The cytoplasm fraction (2000×g supernatant of the homogenates) obtained from liver was solubilized in SDS sample buffer and 20 µg protein was electrophoresed through 15% SDS-polyacrylamide gels. Proteins were blotted onto PVDF membranes and probed with antisera specific for rat HO-1. Lower panel: A duplicate membrane was probed with antisera to rat p53 as a radiation-induced positive control.
The protein level of p53 increased 2 h after irradiation, and then gradually decreased up to 10 h (Fig. 4, lower panel). These results confirmed that the HO-1 mRNA increase was reflected in translation to protein.

In this study we demonstrated that ionizing radiation is a stressor which induces activation of the HO-1 gene and increases enzyme activity in the liver. The acute enhancement of transcription of the HO-1 gene in liver was transient (2 to 4 h after irradiation). However, Deev et al.\(^6\) reported an enhancement of heme oxygenase activity at 5 to 7 days after X-irradiation. It is known that lipid peroxidation is induced in the liver by radiation, as detected by the TBARS method\(^17\), and that one of the products of membrane lipid peroxidation, 4-hydroxynonenal, is known to induce HO-1 gene expression\(^18\). Therefore, we suggest that the increase of HO-1 activity at 5 to 7 days reported by Deev et al. might have been induced by oxygen stress-mediated products, such as 4-hydroxynonenal. It had been reported that the transcription of the HO-1 gene is enhanced by UVA in cultured human skin fibroblasts, and that an antioxidant, N-acetylcysteine, suppressed HO-1 induction\(^19\). Therefore, ROS are presumed to be involved in the gene activation of HO-1 by UVA. It is possible that the products of the heme oxygenase reaction, biliverdin and bilirubin, might scavenge ROS produced by radiation to protect tissues from oxidative stress, and might contribute to some extent to attenuating the secondary injuries. Additionally, heme oxygenase-1 might also contribute to ameliorate radiation-induced injuries in different manners, for example via the carbon monoxide-sGC pathway\(^1\).

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