ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 4, 2004

Forum Original Research Communication

Enhanced Plasminogen Activator Inhibitor-1 Expression in Transgenic Mice with Hepatocyte-Specific Overexpression of Superoxide Dismutase or Glutathione Peroxidase

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ABSTRACT

In this study, we developed a double-transgenic mouse model allowing hepatocyte-specific and regulated expression of the redox-modifying enzymes copper/zinc superoxide dismutase (SOD) and glutathione peroxidase (GPX) by using a tetracycline-regulatable gene expression system. Within this system, the SOD and GPX level can be regulated deliberately by addition or removal of doxycycline hydrochloride to the drinking water. As reactive oxygen species (ROS) have been implicated in a number of pathological conditions, such as atherosclerosis, thrombosis, or liver fibrosis, processes that are also frequently associated with enhanced levels of plasminogen activator inhibitor-1 (PAI-1), it was the aim of the present study to investigate the influence of SOD and GPX overexpression on the regulation of PAI-1. PAI-1 mRNA and protein levels in tetracycline transactivator-dependent SOD-overexpressing double-transgenic mice reached values 2.5- to threefold above the normal mRNA level. By applying doxycycline, a deinduction of the PAI-1 levels was observed. By using the same protocol, PAI-1 mRNA and protein levels were enhanced in GPX double-transgenic mice, and again this response was blunted by the addition of doxycycline. These studies provide some new informaton regarding the role of ROS within the proteolytic processes in hepatocytes that require PAI-1. *Antioxid. Redox Signal.* 6, 721–728.

INTRODUCTION

FIBROSIS OF THE LIVER is associated with many chronic diseases and involves the response to a wide variety of insults believed to enhance formation of reactive oxygen species (ROS). Meanwhile, ROS have been implicated to play an important role in the regulation of a number of cellular processes, including proliferation, coagulation, fibrinolysis, and fibrosis (15, 23), which are primarily controlled via the plasminogen/plasmin system. Within this system, the inactive zymogen plasminogen is converted to the active endopeptidase plasmin by the tissue-type and urokinase-type plasminogen activators (tPA and uPA) (38). The tPA and uPA activity is regulated, in part, by plasminogen activator inhibitors (PAI) (37) from which PAI-1 is the primary physiological inhibitor (20). It can be produced by platelets, vascular endothelial cells (19), vascular smooth muscle cells (43), and several nonvascular cell types, among them hepatocytes (9, 28). Thus, PAI-1 appears to play a key role in the control of fibrinolysis as well as in the development of atherosclerosis or progressive liver fibrosis (2, 18, 30, 39) via the indirect inhibition of the broad-spectrum protease plasmin.

Many pathological conditions, such as atherosclerosis or fibrosis, under which high PAI-1 levels can be found are also associated with enhanced levels of ROS, such as superoxide anion radicals ($O_2^{-\cdot}$) or hydrogen peroxide (H_2O_2). Normally, cellular levels of ROS are usually tightly controlled and dismutation of $O_2^{-\cdot}$ either occurs spontaneously at low pH or is

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catalyzed by members of the superoxide dismutase (SOD) family, thus forming H_2O_2. H_2O_2 is then degraded by glutathione peroxidase (GPX) in the cytosol and mitochondria or by catalase in peroxisomes (12). However, although considerable effort has been made to create appropriate models to elucidate fibrogenic mechanisms, until now no attempt was made to unravel the relationship between ROS and PAI-1 expression in the liver *in vivo.*

Therefore, we aimed to generate a mouse model with conditional hepatocyte-specific expression of the cytosolic copper/zinc SOD (Cu/Zn-SOD). To circumvent any deleterious effects that may occur during development, we used a tetracycline (Tet) regulatory system (25, 26, 46) composed of two elements, namely, the Tet-controlled transactivator (tTA) and the bidirectional tTA-responsive promoter (tTA-RE) (4, 25). The tTA-RE promoter drives the expression of the target gene only in the absence of Tet or its derivative doxycycline (Dox) (3) because in the presence of Dox, tTA is inactive and cannot bind to the tTA-RE promoter, and thus the target gene is silenced. In the present study, the bidirectional tTA-RE promoter is used to direct the simultaneous expression of two proteins, either luciferase (Luc) and Cu/Zn-SOD or Luc and GPX (Fig. 1). The tissue specificity is provided by the promoter controlling the expression of tTA, and hepatocyte-specific expression was achieved by using the albumin (Alb) promoter in context with its enhancer (Alb-P/E). Functional double-transgenic mice were obtained by breeding the liver-specific tTA mouse (Alb-P/E-tTA) with transgenic animals in which the expression of either Cu/Zn-SOD or GPX was directed by the tTA-RE promoter (Fig. 1). Thus, our model allows the study of PAI-1 expression in hepatocytes *in vivo* by controlled overexpression of Cu/Zn-SOD and GPX, and we report here a direct relationship to PAI-1 expression.

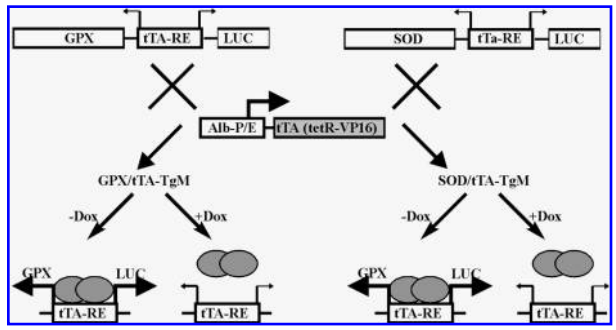

FIG. 1. Schematic outline of the conditional SOD and GPX expression by using the Tet-regulatable system in double-transgenic mice. Transgenic mice harboring the Cu/Zn-SOD and the Luc gene or the GPX and the Luc gene under the control of the biTet promoter were generated. The biTet promoter contains the Tet- or tTA-responsive element tTA-RE, which consists of seven copies of the 42-bp Tet operator sequence between two enhancer-lacking CMV promoters (P_{minCMV}, indicated by arrows), thus keeping the P_{minCMV} silent. Activation of the promoter can only be achieved by binding of the tTA to the tTA-RE in the absence of the Tet derivative Dox. The tTA is a fusion product of the tet-repressor (tetR) and the herpes simplex viral VP16 activation domain. Tissue and cell specificity is provided by the albumin promoter- and enhancer-driven tTA transgenic mice (Alb-P/E-tTA). Only in hepatocytes of double-transgenic mice (SOD/tTA-TgM or GPX/tTA-TgM), the tTA can bind to the tTA-RE, thus activating SOD and Luc or GPX and Luc transcription, respectively. As Dox is added to the drinking water, binding of the tTA is lost and transcription from the tTA-RE is turned off. TgM, transgenic mouse.

EXPERIMENTAL PROCEDURES

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers.

Plasmids

The mouse GPX gene was cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) after PCR amplification from genomic DNA with the sense primer 5′-AGTACGGATTCCACGTTTGAGTCC-3′ and the antisense primer 5′-ACAGGGAGAAAGAAAAGCATAAGG-3′. The EcoRV/ SpeI GPX fragment was cloned into the EcoRV/SpeI sites of pBSK biTet-Luc-pA.

The vector pBSK-biTet-Luc-pA was generated by cloning the EcoRI fragment with the bidirectional Tet (biTet) operator responsive promoter from pBI-1 (25) into pBSK-Luc-pA, which was generated by ligating the XhoI/SalI fragment from pGL3 basic (Promega, Heidelberg, Germany) containing the Luc gene and the SV40 late poly A sequences into the XhoI site of pBluescript-SKII (Stratagene, Heidelberg, Germany).

The mouse Cu/Zn-SOD cDNA was amplified by RT-PCR with the 5′-AAGCATGGCGATGAAAGCGGT-3′ sense and 5′-AATGTTTACTGCGCAATCCCAATC-3′ antisense primers and T/A-cloned into pGEM-T-Easy (Promega) from which a 500-bp EcoRI fragment encompassing the entire SOD cDNA was excised, blunted with Klenow fragment, and ligated into the SmaI/EcoRV sites of pBSK-biTet-Luc-pA. To generate a polyadenylation site after the SOD cDNA, a bovine growth hormone (bGH-pA) XbaI fragment from pUChbGH-pA was cloned into the XbaI sites to give pBSK biTet-Luc-SODbGH-pA. The pUChbGH-pA was generated from ligating the bGH-pA XhoI fragment from pRCCMV into the SalI site of pUC19.

To generate a hepatocyte-specific and Tet regulatable expression of the SOD or GPX gene, it was necessary to express the Tet repressor (tTA) in a hepatocyte-specific manner. To do this, the tTA gene encoding a Tet repressor VP16 fusion should be expressed under the control of the albumin enhancer and albumin promoter. Therefore, the albumin enhancer 5′ primer TGGAGCTTACTTCTTTGATTTGAT and the albumin enhancer 3′ primer AGACATGCCTGATTGGTAGC were used to amplify a 772-bp fragment from genomic DNA, which was T/A cloned in pCR2.1 to give pAlb-enh.

The albumin promoter vector (pCR2.1 pAlb-prom) was cloned in a similar way by using the 5′ primer 5′-CAAGGCCCACACTGAAATGC-3′ and the 5′-GTGTCGAGAAAGACTCG-3′ primer, respectively.

In the next step, the vector pUHD15–1neo (25) containing the tTA gene as tetR-VP16 fusion was cut with EcoRI/BamHI, and the excised tTA gene was cloned into pUChbGH-pA to give pUC-tTA-bGH-pA. Then the albumin enhancer and the promoter were cloned in front of the tTA gene. This was done by excising the EcoRI fragments from pCR2.1 Albprom and pCR2.1 Albenh and simultaneously ligating

them into the EcoRI site of pUChbGH-pA to give pAlbprom/enh-tTA-bGH-pA.

Generation of transgenic mice

The ClaI/PvuII DNA fragment from pBSK biTet-Luc-SODbGH-pA and pAlbprom/enh-tTA-bGH-pA and the NotI/BamHI fragment from pBSK pbiTet-Luc-GPX were gel-purified for microinjection. The constructs were injected into FVB fertilized mouse eggs to generate transgenic mice as described (29, 46).

Positive animals were identified by analyzing DNA from mouse tail (DNeasy tissue kit; Qiagen, Hilden, Germany) by PCR using specific primers. For identification of the Alb-P/E-tTA transgenic animals, the albumin promoter primer 5′-CAAGGCCCACACTGAAATGC-3′ was used as a sense primer and the 5′-GCAAAAGTGAGTATGGTGCC-3′ tTA oligonucleotide as an antisense primer. To identify the transgenic mice harboring the biTet-Luc-SODbGH-pA or biTet-Luc-GPX transgene, the oligonucleotides 5′-GGCGTG-TACGGTGGGAGG-3′ from the biTet promoter and from the Luc gene 5′-GCAATTGTTCCAGGAACCAGGGCG-3′ were used as sense and antisense primers, respectively. Nontransgenic breeder mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Animal experiments were performed in accordance with the European Council Directive of November 24, 1986 (86/609/EEC) and were approved by the local authorities (Bez.-Reg. Braunschweig, 509.42502/01–35.98, 21.12.1998). Mice were kept on a 12-h day/night rhythm with free access to water and food (Altromin 1324; Altromin Gesellschaft für Tierernährung, Lage, Germany). Double-transgenic animals containing the biTet-Luc-SOD or biTet-Luc-GPX transgenes and expressing tTA in hepatocytes were obtained by intercrossing individuals of both lines each.

To omit expression of SOD or GPX in the embryos, a 5% aqueous sucrose solution containing Dox (50 μg/ml; Sigma, Taufkirchen, Germany) was supplied during pregnancy. SOD or GPX overexpression was then gained by replacing Dox with water.

Preparation of cell extracts and Luc activity assay

Mice were anesthetized with pentobarbital (60 mg/kg of body weight) prior to preparation of livers, which had been removed and from a small piece of which cell lysates were prepared as described (5). In brief, liver tissue was transferred to a 15-ml glass centrifuge tube before it was homogenized on ice with an Ultraturrax (Jahnke and Kunkel, Staufen, Germany) for 25 s. The homogenate was then transferred to an Eppendorf cup and centrifuged for 20 min at 14,000 rpm in a Sorvall SS34 rotor at 4°C. The detection of Luc activity in 1 μg of the lysates was performed with the Luciferase Assay Kit (Berthold, Pforzheim, Germany) according to the instructions given by the supplier.

RNA preparation and northern analysis

Isolation of total RNA and northern analysis were performed as described (35). Digoxigenin (DIG)-labeled antisense RNAs served as hybridization probes; they were generated by *in vitro* transcription from pBS-PAI-1, pGEM-SOD, and pBS-GPX using T3 RNA polymerase or from pBS-β-actin using T7 RNA polymerase and RNA labeling mixture containing 3.5 m*M* 11-DIG-UTP, 6.5 m*M* UTP, 10 m*M* GTP, 10 m*M* CTP, and 10 m*M* ATP. Hybridizations and detections were carried out essentially as described before (35). Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen, Germany).

Western blot analysis

PAI-1, SOD, and GPX western blot analysis was carried out as described (31, 56). In brief, protein lysates were collected and the protein content was determined using the Bradford method. Fifty micrograms of protein was loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel and after electrophoresis blotted onto nitrocellulose membranes. The primary rabbit antibody against rat PAI-1 (American Diagnostics) was used in a 1:200 dilution. The secondary antibody was a goat anti-rabbit IgG (Santa Cruz Biotechnology) and used in a 1:2,000 dilution. The monoclonal antibody against Cu/Zn-SOD (Calbiochem) was used in a 1:1,000 dilution. The antibody against GPX was generated by immunizing rabbits with purified GPX using standard techniques and was used in 1:500 dilution. A goat anti-mouse and a goat anti-rabbit immunoglobulin G (Calbiochem) were used as secondary antibodies. The primary rabbit antibody against Golgi membrane (Bioscience, Götingen, Germany) was used in a 1:8,000 dilution. The secondary antibody was a goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany), used in a 1:2,000 dilution. The ECL western blotting system (Amersham, Freiburg, Germany) was used for detection.

RESULTS

SOD and GPX overexpression does not impair embryonic development

From 30 founder animals carrying the biTet-Luc-SOD transgene and from 25 biTet-Luc-GPX founders, three lines each were established and crossed with Alb-P/E-tTA animals from which only one line could be established. Intercrosses of biTet-Luc-SOD or biTet-Luc-GPX mice with Alb-P/E-tTA mice were performed in order to get SOD/tTA or GPX/tTA double-transgenic mice. The crossing experiments were performed either in the presence of Dox, to avoid expression of the transgene in fetal double-transgenic animals, or in control groups in the absence of Dox, to investigate whether overexpression of either SOD or GPX in the fetal liver may affect outcome of the offspring. In both cases, it appeared that SOD or GPX overexpression in the double-transgenic SOD/tTA or GPX/tTA fetuses did not impair normal fetal development and outcome of the pregnancy. Further, in the Dox control group with nontransgenic siblings, no toxic effects of Dox *per se* were observed.

Dox-dependent Luc expression in the livers of transgenic mice

Eight weeks after birth of the double-transgenic mice, Dox was removed from the drinking water for 3 days, and liver samples were prepared and checked for Luc expression in

comparison with samples from mice further receiving Dox or wild-type control animals. It was found that removal of Dox in the SOD/tTA mice enhanced Luc activity by ~2.5-fold compared with the Dox group or the wild-type animals (Fig. 2).

In the GPX/tTA mice, the removal of Dox had similar effects. The Luc activity in the GPX/tTA mice was about twofold higher than in the Dox group or the wild-type mice (Fig. 2).

These data indicate that the Luc gene expressed from the biTet-responsive promoter could be regulated in a Dox-dependent manner in the double-transgenic mice and suggest that both transgenes SOD and GPX may be regulated in the same way.

Dox-dependent PAI-1 mRNA expression in the livers of transgenic mice

To investigate whether the overexpression of SOD or GPX may have any impact on the expression of a putative ROS target gene, the PAI-1 mRNA levels were measured.

From the livers of 8-week-old double-transgenic mice that did not receive Dox within the drinking water for 3 days, total RNA was prepared, and the levels of PAI-1 mRNA were measured by northern blot hybridization and compared with the PAI-1 mRNA levels from samples of mice further receiving Dox or of wild-type control animals. The PAI-1 mRNA was weakly expressed in the livers of wild-type animals and the Dox-receiving SOD/tTA mice. Upon removal of Dox, the PAI-1 mRNA level increased between 2.5- and threefold in the SOD/tTA mice when compared with the Dox group or the wild-type animals (Fig. 3).

Interestingly, in the GPX/tTA mice, the overexpression of GPX by removal of Dox had similar effects. Whereas PAI-1 mRNA in the control Dox-receiving group and in the wild-type animals remained expressed at a relatively low level, the absence of Dox again permitted an induction of PAI-1 mRNA by about twofold (Fig. 3).

Thus, overexpression of both redox-modifying enzymes mediated an enhanced expression of PAI-1 mRNA.

SOD, GPX, and PAI-1 protein expression can be modulated by Dox in the livers of transgenic mice

To investigate whether the Dox-regulatable SOD or GPX expression occurs also on the protein level and whether the SOD- and GPX-mediated enhancement of PAI-1 mRNA has a

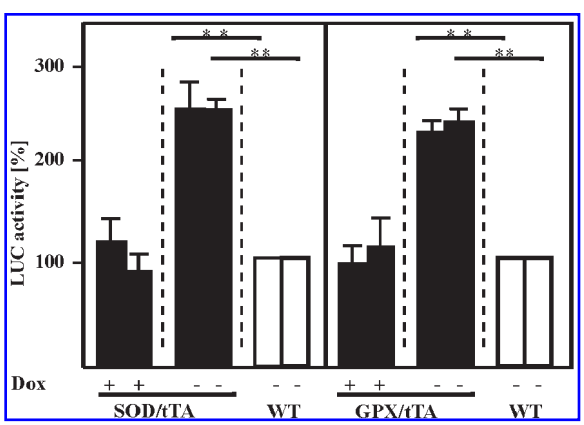

FIG. 2. Enhanced Luc activity in the livers of double-transgenic mice. Transgenic mice harboring the Cu/Zn-SOD and the Luc gene or the GPX and the Luc gene under the control of the biTet promoter were crossed in the presence of Dox with transgenic mice expressing the tTA under the control of the albumin promoter enhancer. All double-transgenic mice (SOD/tTA and GPX/tTA) and a wild-type (WT) control group received Dox until the age of 8 weeks. Then Dox was removed from the drinking water for 3 days in one group each of SOD/tTA and GPX/tTA mice. Livers were removed, and Luc activity was determined (see Materials and Methods). In each experiment, the Luc activity measured in the wild-type mice was set equal to 100%. Values are means ± SE of three independent experiments with two animals each. Statistics using Student's *t* test for paired values: **significant differences WT versus SOD/tTA without Dox or GPX/tTA, $p \leq 0.05$.

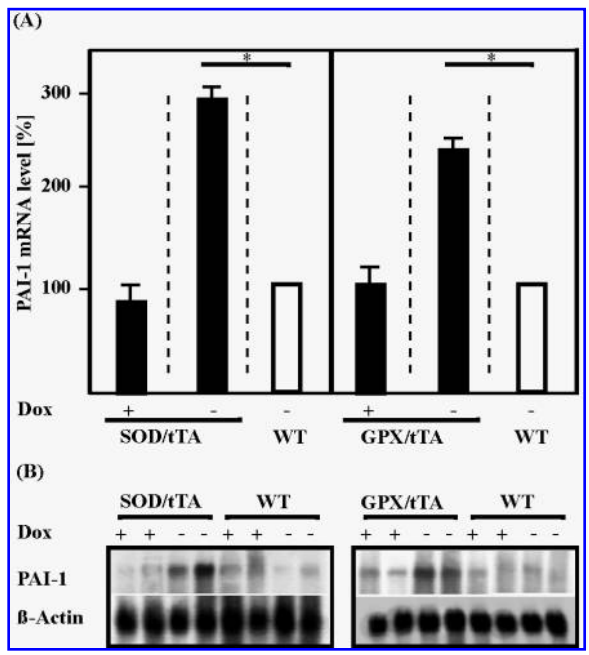

FIG. 3. Enhanced PAI-1 mRNA expression in the livers of double-transgenic mice. Double-transgenic mice expressing the Cu/Zn-SOD and the GPX in a tTA-dependent manner were generated and maintained as in Fig. 1. Dox was removed from the drinking water for 3 days in one group each of SOD/tTA and GPX/tTA mice. Livers were removed, and total RNA was prepared and analyzed (see Materials and Methods). (**A**) In each experiment, the PAI-1 mRNA levels measured by northern blotting (see B) in the wild-type mice were set equal to 100%. Values are means ± SE of three independent experiments with two animals each. *Significant differences WT versus SOD/tTA without Dox or GPX/tTA, $p \leq 0.05$. (**B**) Representative northern blot. For northern analysis, 15 μg of total RNA was hybridized to DIG-labeled PAI-1 and β-actin antisense RNA probes (see Materials and Methods). Signals were obtained by chemiluminescence and scanned by videodensitometry.

functional consequence on the protein level, we studied SOD, GPX, and PAI-1 expression by western blot analysis.

From pieces of the same livers that were analyzed for Luc activity and PAI-1 mRNA expression, total protein was prepared and subjected to western analysis. When the SOD protein levels were measured, it was found that the Dox control group expressed about the same level on SOD protein as the wild-type mice with or without Dox (Fig. 4). By contrast, when Dox was omitted for 3 days, the SOD level increased by about threefold when compared either with the controls or with the wild-type mice. In concordance with the results from the PAI-1 northern blots, the PAI-1 protein level was increased between 2.5- and threefold in the SOD/tTA mice upon removal of Dox, whereas no changes were detected in the Dox-receiving group and the wild-type animals (Fig. 4).

Similarly, in the GPX/tTA mice, the presence of Dox prevented an increase in the GPX protein levels, however, removal of Dox for 3 days mediated an about threefold enhancement of GPX protein levels when compared with the wild-type mice. Again, concomitant with the PAI-1 mRNA, no changes in PAI-1 protein level were detected in the control Dox-receiving group and in the wild-type animals. Here the PAI-1 protein remained expressed at a relatively low level, but as seen with the PAI-1 mRNA, the absence of Dox again permitted an induction of PAI-1 protein by about threefold in comparison with the wild-type mice (Fig. 4).

Thus, in the model with double-transgenic SOD/tTA and GPX/tTA mice, the controlled overexpression of both SOD and GPX mediated an enhanced expression of PAI-1.

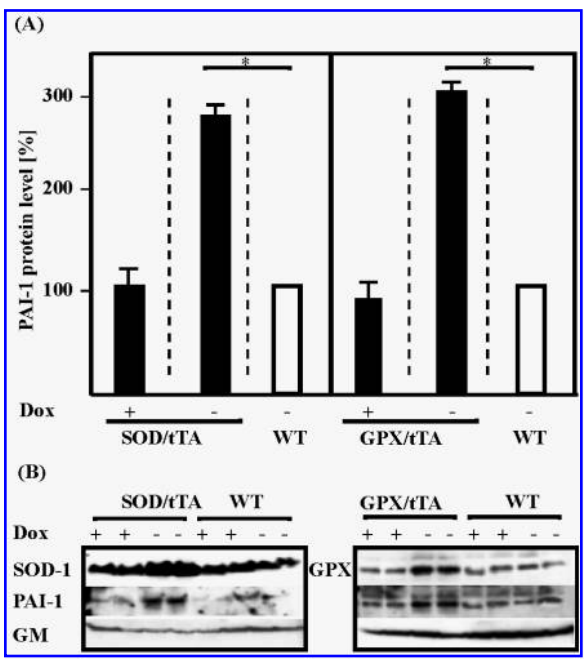

FIG. 4. Induction of PAI-1, SOD, and GPX protein expression in the livers of double-transgenic mice. Dox was removed from the drinking water for 3 days in one group each of double-transgenic SOD/tTA and GPX/tTA mice. Livers were removed, and protein was prepared and analyzed (see Materials and Methods). (**A**) In each experiment, the PAI-1 protein levels measured by western blotting (see B) in the wild-type mice were set equal to 100%. Values are means ± SE of three independent experiments with two animals each. *Significant differences WT versus SOD/tTA without Dox or GPX/TA, $p \leq 0.05$. (**B**) Representative western blot. For western blot analysis, 50 μg of protein was subjected to western analysis (see Materials and Methods) with antibodies against PAI-1, SOD, GPX, and Golgi membrane (GM). Signals were obtained by chemiluminescence and scanned by videodensitometry.

DISCUSSION

In the present study, a double-transgenic mouse model allowing hepatocyte-specific and Tet-regulated expression of the redox-modifying enzymes Cu/Zn-SOD and GPX was developed to study PAI-1 expression, which has been implicated in a number of pathological conditions frequently associated with ROS. Within this model, we could show that PAI-1 mRNA and protein levels were enhanced in both the SOD/tTA and the GPX/tTA double-transgenic mice. By applying Dox, a repression of the PAI-1 levels was observed. Thus, these studies may provide some mechanistic details for the involvement of ROS within the PAI-1 requiring proteolytic pathological processes such as liver fibrosis.

ROS as modulators of PAI-1 expression

The result of this study, that modulation of the ROS levels *in vivo* by overexpression of the redox-modifying enzymes SOD and GPX can modulate PAI-1 expression, appears to be consistent with a number of *in vitro* studies showing the role of ROS in the regulation of PAI-1 gene expression in different cell types. In most investigations, vascular endothelial cells were used as a model system, but some studies were also performed in kidney tubule epithelial or glomerular mesangial cells and adipocytes. The finding of the present study that enhanced expression of SOD in hepatocytes of the SOD/tTA double-transgenic mice would promote formation of H_2O_2 and enhance PAI-1 mRNA and protein production is in line with experiments performed in primary cultures of human umbilical vein endothelial cells showing induction of PAI-1 by H_2O_2 and hydroxyl radicals (11, 13, 16, 50, 54). ROS formation and PAI-1 induction may occur in response to several environmental, nutrient, hormonal, growth-factor, and cytokine-stimulated processes. For instance, ionizing irradiation of rat kidney tubule epithelial NRK52E cells can lead to production of various radicals, including $O_2^{-\bullet}$ and H_2O_2 together with a concomitant dose-dependent increase in PAI-1 mRNA and protein levels (57). This radiation-induced induction in PAI-1 expression could be inhibited by the antioxidant *N*-acetylcysteine and overexpression of catalase. Interestingly, overexpression of mitochondrial manganese SOD did not affect radiation-induced PAI-1 expression. These results are in line with the present study suggesting that $O_2^{-\bullet}$ or H_2O_2 generated in the cytoplasma contributes to the regulation of PAI-1 expression.

At first glance, it was surprising that within the present study overexpression of GPX in the double-transgenic GPX/tTA mice enhanced PAI-1 production. Several studies implicated that antioxidants such as *N*-acetylcysteine may

counteract the H_2O_2-mediated enhancement of PAI-1 in NRK52K cells (57), human renal proximal tubular cells HK-2 (40), or glomerular mesangial cells (17). Only one study, investigating the pathogenesis of human immunodeficiency virus (HIV) infection, showed a trend toward increasing levels of erythrocyte GPX with increasing PAI-1 levels in HIV patients (52). On the other hand, the overexpression of GPX may account for a similar decrease in the H_2O_2 levels as observed under hypoxia (34). Similarly, this dual response relates to the expression of the heme oxygenase-1 gene, which responds to oxidants, as well as to deviations in oxygen tension such as during hypoxia. Hypoxia has been shown to be a potent activator of PAI-1, as well as of heme oxygenase-1 expression, in different cell types, including primary hepatocytes (35) and HepG2 hepatoma cells (36). Further, in HepG2 cells, treatment with the antioxidant pyrrolidinedithiocarbamate or coexpression of redox factor 1 (Ref-1) enhanced PAI-1 expression (24). Further, in a study performed in the kidney tubule epithelial cells, both PAI-1 and plasminogen activators were shown to be induced by the same ROS-generating stimulus (17), whereas in other studies performed in cardiac microvascular endothelial cells, human umbilical vein endothelial cells, and preadipocytes, no induction or repression was reported (41, 44). Thus, these findings together with the present study underline that the issue of ROS-dependent regulation of PAI-1 expression is still more complicated and may be influenced by *in vitro* versus *in vivo*-like or cell type-specific situations.

ROS and PAI-1 as modulators of liver fibrosis

Oxidative stress has long been known to be involved in the pathogenesis of hepatic fibrosis (21, 42, 51), characterized by the damage of parenchymal cells in the hepatocytes proper, the excessive deposition of extracellular matrix proteins, and remodeling of the liver architecture (1, 8). Meanwhile it became evident that fibrosis reflects an intricate interplay between the hepatocytes and all other nonparenchymal cells, such as Kupffer, perisinusoidal stellate, and endothelial cells together with a number of cytokines and growth factors (21, 42, 51). Within this scenario, the active Kupffer cells, which represent resident macrophages, release ROS and a whole spectrum of different cytokines from which transforming growth factor-β (TGF-β), a potent activator of PAI-1 expression, appears to be very important (6, 47).

The TGF-β-dependent induction of PAI-1 involves a rapid, transient phosphorylation of Smad transcription factors and their direct binding to critical TGF-β-inducible elements in the PAI-1 promoter (14). However, it is noteworthy that also Smad-independent TGF-β responses were observed. They may be mediated by the p38 mitogen-activated protein kinase (55) and the TGF-β-mediated increase in formation of ROS (22), thus initiating an inflammatory phase (27). In line with this, in mesangial cells the TGF-β1- and H_2O_2-induced changes in PAI-1 expression could be reversed by *N*-acetylcysteine (33). Further, the activated Kupffer cells attract cells like polymorphonuclear leukocytes, which then also contribute to hepatocyte damage via direct release of ROS, proteases, and other toxic cytokines such as tumor necrosis factor-α (10, 27), which also has been shown to be able to induce PAI-1 expression via ROS (17). This process with characteristics of an inflammation may be maintained in a vicious cycle by persistent chronic exogenous and endogenous signals. However, fibrogenesis may be reversible as observed in spontaneous regeneration (32). It appeared that during regeneration, degradation of the extracellular matrix containing latent growth factors may require plasminogen activation for activation of latent growth factors such as hepatocyte growth factor (7, 45, 48, 49, 53). As in our system the overexpression of SOD or GPX enhanced PAI-1 expression without cell damage, it suggests that PAI-1 may play also an important role in events closely associated with hepatocyte growth, such as liver regeneration, to prevent an overextensive degradation.

In summary, our findings provide evidence that modulation of the redox state in hepatocytes *in vivo,* either by shifting it to a "prooxidant" state due to overexpression of SOD or by generation of a more reducing environment after overexpression of GPX, enhanced PAI-1 expression. These results demonstrate that a "prooxidant" state with elevated ROS levels as found after liver injury can be associated with remodeling processes requiring the action of PAI-1 to promote these diseases. Thus, shifting the redox balance to reduced conditions may be beneficial in regeneration processes, thus preventing enhanced activation of hepatocyte growth factors.

ACKNOWLEDGMENTS

We thank Dr. T.D. Gelehrter (Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI, U.S.A.) for the kind gift of PAI-1 cDNA and the animal care facility of the Max PIanck Institute for Experimental Medicine for assistance and support with generation of transgenic animals. This study was supported by the Deutsche Forschungsgemeinschaft SFB 402 Teilprojekt A1 and GRK 335.

ABBREVIATIONS

Alb, albumin; biTet, bidirectional tetracycline; Cu/Zn-SOD, copper/zinc superoxide dismutase; DIG, digoxigenin; Dox, doxycycline; GPX, glutathione peroxidase; H_2O_2, hydrogen peroxide; Luc, luciferase; $O_2^{-\cdot}$, superoxide anion radical; PAI, plasminogen activator inhibitor; ROS, reactive oxygen species; SOD, superoxide dismutase; Tet, tetracycline; TGF-β, transforming growth factor-β; tPA, tissue plasminogen activator; tTA, tetracycline transactivator; tTA-RE, tTA-responsive element; uPA, urokinase plasminogen activator.

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Received for publication March 3, 2004; accepted April 19, 2004.

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