

Stat3 confers resistance against hypoxia/reoxygenation-induced oxidative injury in hepatocytes through upregulation of Mn-SOD

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Background/Aims: Hypoxia/reoxygenation (H/R) causes oxidative stress to the cell and induces apoptotic cell death. Signal transducer and activator of transcription-3 (Stat3) is one of the most important molecules involved in the initiation of liver development and regeneration, and has recently been shown to protect cells against various pathogens. In order to investigate the hepatoprotective effects of Stat3, we examined whether it protects against H/R-induced injury in primary hepatocytes.

Methods: Primary cultured hepatocytes were prepared from SD rats. Adenoviruses and cytokines were added 2 days and 1 h prior to the H/R insult, respectively. Hepatocytes and culture media were harvested for the assays before and after H/R insult.

Results: Interleukin-6 and cardiotropin-1, which may function mainly through Stat3 activation, protected cells from H/R-induced apoptosis. Adenoviral overexpression of the constitutively activated form of Stat3 (Stat3-C) reduced H/R-induced apoptosis as well as generation of reactive oxygen species (ROS) in hepatocytes. Interestingly, Stat3-C induced Mn-SOD, but not Cu/Zn-SOD, both at the protein and mRNA levels. Overexpression of Mn-SOD significantly reduced H/R-induced ROS and apoptosis by inhibiting redox-sensitive activation of caspase-3 activity.

Conclusions: Stat3 protects hepatocytes from H/R-induced cell injury at least partly by upregulating Mn-SOD and inactivating caspase-3.

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Keywords: Hypoxia/reoxygenation; Oxidative stress; Stat3; Mn-SOD; Apoptosis; Hepatocyte

Received 27 March 2004; received in revised form 20 July 2004; accepted 18 August 2004; available online 15 September 2004

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1. Introduction

Ischemia-reperfusion (I/R) injury plays a critical role in various clinical disorders, such as cerebral and myocardial infarction. In liver, I/R-induced injury is mainly caused by surgical intervention, such as hepatectomy and liver transplantation. Post-operative complications caused by I/R are still serious concerns in liver surgery [1]. Therefore, establishing an efficient strategy to combat I/R injury is a critical issue in order to reduce these complications. Hepatic I/R results in massive

cell death accompanied by neutrophil infiltration, but its pathophysiological mechanisms remains unclear [2]. According to numerous studies of hypoxia-reoxygenation (H/R), which is a simple *in vitro* model of I/R, tissue (cell) injury at early post-H/R periods is mediated by the cellular generation of reactive oxygen species (ROS) [3]. One of the major sources of cellular ROS generated immediately after H/R may be functionally impaired mitochondria [4,5]. Excessive ROS not only exert direct effects on cells through lipid peroxidation, protein degradation and DNA damage, but also indirectly by regulating redox-sensitive signals which may affect cell fate and inflammation [6]. Redox-sensitive signaling molecules such as NF- κ B, AP-1, and some MAPKs are currently considered to be activated under these conditions [7]. A number of trials to reduce H/R-induced cell death have been performed [8–11], but there is no conclusive consensus.

Signal transducer and activator of transcription-3 (Stat3) is a latent cytoplasmic transcription factor that functions as an intracellular effector activated by various ligands including members of the interleukin-6 (IL-6) family, EGF and PDGF, and by a number of receptor- and nonreceptor-tyrosine kinases [12–16]. Stat3 was originally investigated as a molecule that was capable of selectively interacting with an enhancer element in the promoter of acute-phase response genes following stimulation by interleukin-6 (IL-6) [17,18]. Stat3 activation requires tyrosine-phosphorylation induced by cytokine receptor-associated kinases (Jak), growth factor receptor-tyrosine kinases, or nonreceptor-tyrosine kinases such as Src and Abl. Activated Stat3 forms homo- or hetero-dimers by interactions between reciprocal pY-SH2 domains, translocates to the nucleus, and binds to the specific promoters of its target genes to induce gene expression [19]. Physiological functions of Stat3 have been extensively studied, and it is known to play crucial roles in organ development and cell proliferation [16]. The studies using liver-specific conditional knockout mice have indicated that Stat3 is an important initiator of hepatocellular proliferation [20–22].

Other than cell proliferation, a cellular protective function of Stat3 has been recently reported in a variety of cells. Stat3 activation protects hepatocytes against Fas-induced apoptosis [23], fibroblasts against apoptosis induced by serum-withdrawal or UV [24], cardiomyocytes against H/R-induced cellular damage [25], and leads to T cell proliferation by preventing apoptosis [26]. Additionally, there is evidence that Stat3 confers anti-apoptotic properties to various types of cancer cells [27–29].

However, it is still unclear whether Stat3 is involved in hepatocellular protection against H/R-induced, ROS-mediated injury. The present study was designed to investigate the protective effect of Stat3 against H/R-induced oxidative stress in hepatocytes and its underlying mechanism. Especially, involvement of anti-oxidant enzymes in the protection against H/R-induced injury was studied to elucidate the anti-apoptotic mechanism in a redox-dependent manner.

2. Materials and methods

2.1. Adenoviral vectors

A cDNA construct of *Stat3-C* (constitutively active form of Stat3), which was provided by Dr James E. Darnell (Rockefeller University, New York, USA), was made by substituting cysteine residues for A661 and N663 of murine Stat3 and was tagged with FLAG. This renders the Stat3-C molecule capable of dimerizing without phosphorylation on Y705 [19]. A replication-deficient adenoviral vector encoding *Stat3-C* (AxCAS3-C) was constructed as previously described [23]. AdLacZ, an adenovirus encoding inert bacterial β -galactosidase, was used as a control vector. Adenoviral vector coding cDNA of manganese superoxide dismutase (AdMn-SOD) was previously described [10].

2.2. Preparation of primary cultures of hepatocytes

Primary cultured hepatocytes were prepared by a conventional collagenase perfusion method from rat liver (Sprague-Dawley, 230–250 g, male). Hepatocytes were seeded at 3×10^6 cells per 10-cm plastic dish coated with rat tail collagen and cultured with William's E complete medium (Gibco, NY) supplemented with 10% fetal bovine serum, 10^{-9} mol/l insulin and 10^{-9} mol/l dexamethasone in a humidified atmosphere of 5% CO₂/95% air at 37 °C. All animals were handled according to uniform policies set forth by the Animal Care and Use Committee of the National Research Institute for Child Health and Development.

2.3. Stat3 induction in hepatocytes and H/R experiments

Ten nanogram per milliliter of IL-6 or cardiotropin-1 (CT-1) (R & D Systems, MN), or 100 μ M AG490 (Calbiochem, CA) were added to the culture medium 1 or 2 h prior to the H/R insult, respectively, to activate Stat3. Adenoviral vector (AdLacZ or AxCAS3-C) was transfected at 2 MOI (multiplicity of infection: the average number of phage particles that infect a single cell) 48 h prior to H/R. Hypoxic conditions were maintained in a modulator incubator chamber (Billups-Rothenberg, CA) by flushing with a 95% N₂/5% CO₂ gas mixture for 10 min and sealing the chamber. This method has been shown to achieve a pO₂ of 10 ± 5 Torr. Following 4-hr hypoxia, reoxygenation of hepatocytes was obtained by opening the chamber and by replacing the hypoxic medium with oxygenated medium.

2.4. Estimation of H/R-induced cellular damage

To evaluate apoptosis, staining with annexin V was performed using the Vybrant™ Apoptosis Assay Kit #3 (Molecular Probes, Eugene) according to the manufacturer's instructions. Briefly, hepatocytes were grown in glass-bottomed dishes (Matsunami Glass Ind., Ltd, Osaka) coated with collagen. After the H/R insult, the cells were washed with phosphate-buffered saline (PBS) and incubated with 5% FITC annexin V and 1 μ g/ml propidium iodide (PI) for 30 min at room temperature. After rinsing with PBS, the hepatocytes were observed with a confocal laser scanning microscope (Fluoview FV500, Olympus, Tokyo) at wavelengths of 480 nm (excitation) and 530 nm (emission). For quantitative evaluation of apoptosis, an enzyme-linked immunosorbent assay (ELISA) kit (Cell Death Detection ELISAPLUS; Roche, Basel) was used according to the manufacturer's instructions.

2.5. Western blot analysis

Fifty micrograms of protein was loaded and run on 15% SDS-PAGE. The protein transferred to the nitrocellulose membrane was incubated with appropriate antibodies: anti-Stat3, anti-phospho-Stat3, anti-Cu/Zn-Superoxide dismutase (SOD), anti-Bcl-2, anti-Bcl-xL, anti-Survivin, anti-A1, anti-Redox factor-1 (Ref-1) (Santa Cruz Biotechnology Inc., Santa Cruz), anti-caspase-3 (cleaved), anti-caspase-9 (Cell Signaling Technology Inc., Beverly), anti-Mn-SOD, anti-Thioredoxin (TRX) (BD Biosciences, Franklin Lakes), anti-Glutathione peroxidase (GPx) (Abcam, Cambridge-shire), anti-FLAG (Sigma-Aldrich, St Louis), and anti-Tubulin (Oncogene Research Products, San Diego).

2.6. Electrophoretic mobility shift assay (EMSA)

Stat3 DNA binding activity was assayed by using the ^{32}P -labeled SIE (sis-inducible element) –m67 oligonucleotide as a probe (5'-actgG-GATTTTCCCGTAAATGGTC-3'). Nuclear protein was electrophoresed as described previously [23].

2.7. RT-PCR assay

Total RNA was extracted using an Isogen reagent (Nippon Gene, Tokyo). First-strand cDNA synthesis was performed with reverse transcriptase, 5 μg of total RNA, and oligo (dT) primers. The cDNA was amplified by PCR with rat Mn-SOD, Cu/Zn-SOD and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer pairs of Mn-SOD and GAPDH were 5'-GCCTGCTCTAATCACGACC-3' and 5'-ATGACAGT-GACAGCGTCCAA-3', and 5'-TCCTGCACCACCAACTGCTTAG-3' and 5'-CAGATCCACAACGGATACATTGG-3', respectively. The thermocycling parameters began with 94 °C for 2 min followed by 25 cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s). PCR products were separated on 1% agarose gels.

2.8. Measurement of cellular reactive oxygen species generation after H/R

Intracellular ROS production of hepatocytes was identified by confocal laser scanning microscopy after staining with 2',7'-dichlorofluorescein (DCFH-DA) (Molecular Probes, Eugene) [30]. Briefly, hepatocytes seeded on glass-bottomed dishes were washed with PBS followed by H/R. After incubation with Hank's buffered salt solution containing 1 μM DCFH-DA for 5 min in the dark, cells were observed by confocal laser scanning microscopy (excitation, 488 nm; emission, 530 nm). To minimize photooxidation of DCFH-DA, fluorescent signals were collected with a single rapid scan. To quantify the intracellular ROS generation, fluorescence intensity was measured also by fluorometric plate reader [31]. Briefly, after various oxidative insults, hepatocytes were incubated with DCFH-DA (1 μM) at 37 °C in the dark for 4 h, followed by measurement of fluorescence intensity (excitation, 485 nm; emission, 535 nm) using a Wallac 1420 ARVO-sx Multi-label counter (Perkin-Elmer Life sciences, Wellesley).

2.9. Measurement of Mn-SOD activity

SOD activity was measured using a SOD Assay Kit-WST (Dojindo Laboratories, Kumamoto) according to the manufacturer's instructions [32]. The principle of this test is based on reduction of NBT (Nitro Blue Tetrazolium). The cell suspension was sonicated on ice using a 550 Sonic Dismembrator (Fisher Scientific Co., Tustin). Equal amounts of protein, as determined by the Bradford protein assay (Bio-Rad, Hercules), were applied to the total SOD activity assay. Mn-SOD activity was calculated by addition of 1 mM KCN, which selectively inactivates Cu/Zn-SOD [33].

2.10. Statistical analysis

The results were expressed as means \pm standard error of at least three independent experiments. Fisher's test was used for analysis of differences between multiple groups. *P* values less than 0.05 were considered significant.

3. Results

3.1. Cell damage induced by H/R

To examine whether H/R induces apoptosis in primary hepatocytes, annexin V staining was employed. Although only a small fraction of the hepatocytes were annexin-

positive at 2 h of reoxygenation, most cells became positive after 4 h (Fig. 1(A)). The apoptotic cell death in the H/R group was significantly higher than in the normoxia group after 8 h of reoxygenation (Fig. 1(B)).

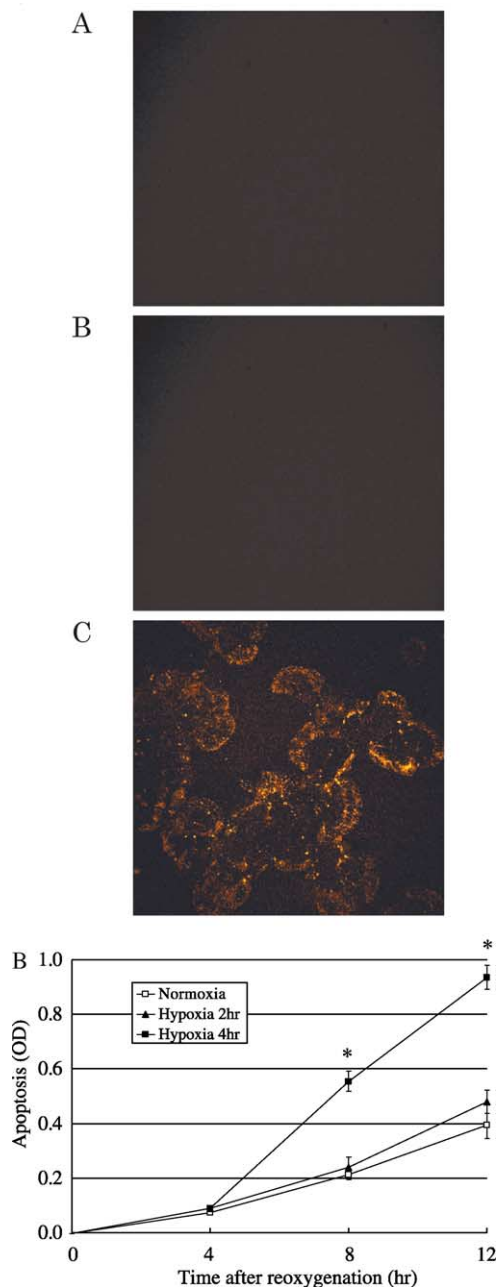


Fig. 1. Hypoxia/reoxygenation-induced cell death in primary hepatocytes. (A) Detection of H/R-induced apoptosis by annexin V staining. Hepatocytes were stained with annexin V (orange) and PI (blue) before hypoxia [A], and 0 [B] and 8 h [C] after reoxygenation, and observed by confocal laser scanning microscopy. Hepatocytes 8 h after reoxygenation shows positive staining of annexin V. Original magnification, $\times 200$. (B) Changes of apoptotic cell death induced by H/R. Following 2 or 4 h of hypoxia, aliquots of the culture media at the time points indicated were used for the apoptosis ELISA. Apoptosis was significantly increased after 8 h following 4 h of hypoxia. **P* < 0.05 versus normoxia group. [This figure appears in colour on the web.]

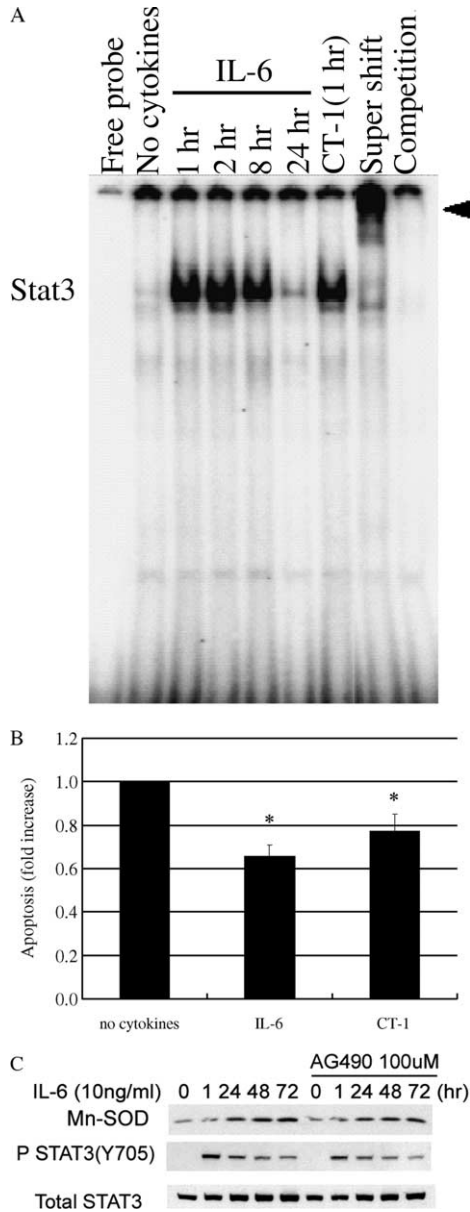


Fig. 2. IL-6 and Cardiotropin-1, Stat3 activators, reduced H/R-induced cell death. (A) Stat3 activation induced by IL-6 or CT-1. Nuclear extracts from hepatocytes treated with 10 ng/ml of IL-6 or CT-1 for indicated durations were incubated with a 32P-labeled SIE-m67 probe and analyzed by electrophoretic mobility shift assay. Stat3 antibody or a 100-fold excess of unlabeled SIE-m67 probe (competition, respectively). The arrowhead indicates a supershifted band. IL-6 and CT-1 induced Stat3 activation at least 1 h after addition, which lasts at least 8 h and ends within 24 h. (B) Protective effects of IL-6 or CT-1 against H/R damage. IL-6 or CT-1 was administered 1 h before hypoxia, and culture media collected 8 h after reoxygenation. Longitudinal axis indicates increase of apoptotic level during 0–8 h after reoxygenation. Numeric values represent the fraction relative to the untreated group (control). Apoptosis was significantly decreased by both cytokines 8 h after H/R. **P* < 0.005 versus no-cytokines group. (C) Inhibition of IL-6-induced Stat3-phosphorylation and Mn-SOD expression by Jak2 inhibitor, AG490. Lysates from hepatocytes treated with IL-6 for indicated duration were analyzed by Western blotting. Stat3 was phosphorylated 1 hr after IL-6 addition and Mn-SOD was induced 24–72 h later. Both of which were inhibited by AG490.

3.2. IL-6 and cardiotropin-1, Stat3 activators, reduce H/R-induced cell death

To elucidate whether Stat3 protects hepatocytes from H/R-induced cell death, cytokines which mainly signal through Stat3 were tested (IL-6 and CT-1). Stat3 activation in hepatocytes by IL-6 or CT-1 was confirmed by EMSA (Fig. 2(A)). Stat3 signals were increased in a dose-dependent fashion within the range of 1–100 ng/ml (data not shown). Fig. 2(B) shows the protective effects of these cytokines on H/R-induced damage. Apoptotic change was significantly reduced compared to control cells without cytokines. These anti-apoptotic effects were observed dose-dependently with maximal effects of both cytokines at 10 ng/ml (data not shown). Additionally, IL-6 immediately

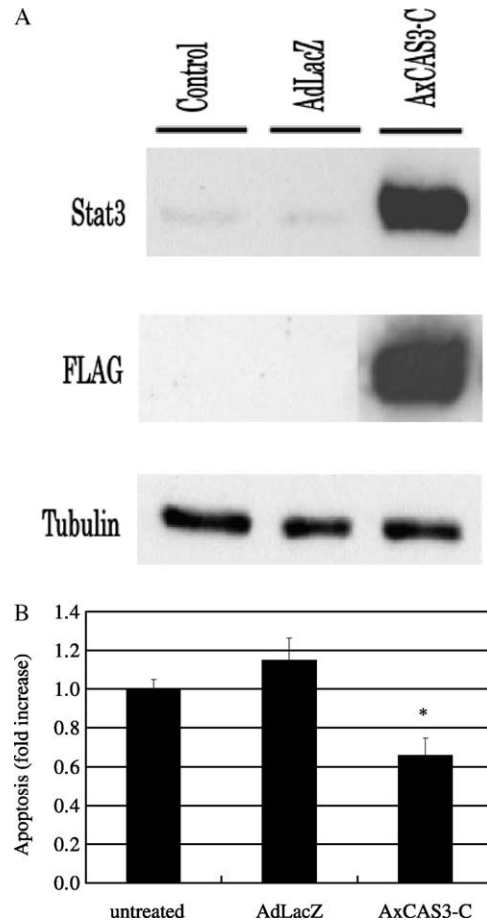


Fig. 3. Adenoviral overexpression of Stat3 protects hepatocytes from H/R-induced cell death. (A) Overexpression of Stat3 by AxCAS3-C transfection. Lysates from hepatocytes transfected with AdLacZ or AxCAS3-C 2 days before were analyzed by Western blotting. Exogenous Stat3 was induced by AxCAS3-C transfection. (B) Protective effects of Stat3 against H/R-induced damage. AdLacZ or AxCAS3-C was transfected 2 days before hypoxia, and culture medium collected 8 h after reoxygenation. Longitudinal axis indicates increase of levels of apoptotic cells during 0–8 h after reoxygenation. Numeric values represent the fraction relative to the untreated group. Apoptosis was significantly decreased by Stat3 overexpression 8 h after H/R. **P* < 0.005 versus AdLacZ group.

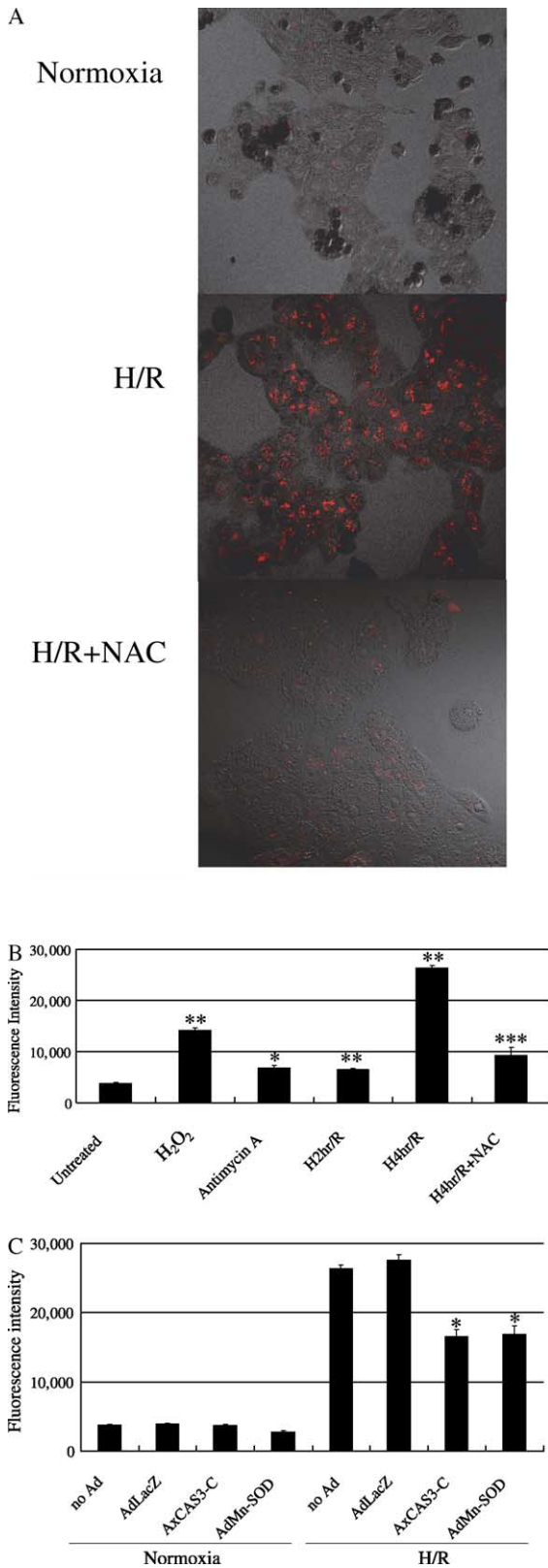


Fig. 4. Stat3-C reduces H/R-induced cellular ROS generation. (A) H/R-induced cellular ROS generation. After H/R treatment, hepatocytes seeded on glass-bottomed dishes were incubated with DCFH-DA (1 μ M) for 5 min in dark, and then analyzed by confocal laser scanning microscopy (excitation, 488 nm; emission, 530 nm). ROS generation was observed after H/R, which was resolved by anti-oxidant. Original

phosphorylated Stat3 and induced Mn-SOD in later time points, which was inhibited by the pre-treatment with AG490, a specific inhibitor of Jak2 (Fig. 2(C)). This suggests the involvement of Jak2 kinase in IL-6-induced and Stat3-mediated Mn-SOD expression.

3.3. Adenoviral overexpression of Stat3 protects hepatocytes from H/R-induced cell death

To elucidate whether Stat3 protects hepatocytes from H/R-induced cell death, an adenoviral vector encoding the active form of Stat3, AxCAS3-C was used. Stat3 was successfully overexpressed by AxCAS3-C gene transfer (Fig. 3(A)). Strongly expressed FLAG protein, tagged with Stat3-C, indicated that overexpressed Stat3 was mostly exogenously introduced. Fig. 3(B) shows the protective effect of exogenously overexpressed Stat3 on H/R-induced damage. Hepatocellular necrosis and apoptosis was slightly increased by AdLacZ transfection (not statistically significant), but a significant decrease of necrosis and apoptosis was demonstrated after AxCAS3-C transfection.

3.4. Stat3-C reduces H/R-induced cellular reactive oxygen species generation

H/R-induced cellular ROS generation was identified by confocal laser scanning microscopy with DCFH-DA staining (Fig. 4(A)). Hepatocytes immediately after hypoxic insult were positively stained with DCFH-DA, which was not detected under normoxic conditions. It was diminished by the addition of the anti-oxidant, *N*-acetyl-L-cysteine (NAC). To quantify H/R-induced cellular ROS, the DCFH-DA fluorescence intensity was measured (Fig. 4(B)). It was significantly increased by the addition of H₂O₂ or antimycin A in a dose-dependent manner (data not shown). H/R insult, especially 4 hr of hypoxia, strongly increased the fluorescence intensity after reoxygenation. This increase was diminished effectively by the addition of NAC.

magnification, $\times 200$. (B) Cellular ROS generation was quantified by using DCFH-DA. After various oxidative insults including H₂O₂, (15 μ M), antimycin A (30 μ M) and H/R, hepatocytes were incubated with DCFH-DA (1 μ M) at 37 $^{\circ}$ C in the dark for 4 h, followed by measurement of fluorescence intensity (excitation, 485 nm; emission, 535 nm). Fluorescence intensity of DCFH-DA was significantly increased by oxidative chemicals and H/R, which was resolved by anti-oxidant. * $P < 0.05$; ** $P < 0.005$ versus no-treatment group; *** $P < 0.005$ versus hypoxia-4 h group. (C) Adenoviral overexpression of Stat3 or Mn-SOD reduces H/R-induced cellular ROS generation. Two days after transfection of AdLacZ, AxCAS3-C or AdMn-SOD, hepatocytes were exposed to H/R, followed by incubation with DCFH-DA and measurement of fluorescence intensity. Increase of ROS generation induced by H/R was decreased by overexpression of Stat3 or Mn-SOD. * $P < 0.05$ versus H/R-AdLacZ group. [This figure appears in colour on the web.]

In order to assess the mechanism of the cytoprotective (anti-apoptotic) effect of Stat3, we next examined the effects of Stat3 and Mn-SOD on H/R-induced ROS generation. Very interestingly, adenovirally overexpressed Stat3 and Mn-SOD both suppressed H/R-induced ROS generation to the same degree (Fig. 4(C)). This finding led us to the idea that Stat3 may suppress H/R-induced injury through upregulation of anti-oxidant molecules such as Mn-SOD.

3.5. Expression of anti-apoptotic and anti-oxidative proteins induced by Stat3-C

To address the mechanism of the redox-dependent protection mediated by Stat3, we next examined expression of anti-apoptotic and anti-oxidant proteins 2 days after-AxCAS3-C transfection (Fig. 5(A) and (B)). This revealed that Survivin and Mn-SOD, were significantly upregulated

by Stat3-C. Interestingly, Mn-SOD, but Cu/Zn-SOD, was specifically expressed (Fig. 5(C)). RNA transcripts of Mn-SOD were also upregulated by Stat3-C though Cu/Zn-SOD was constitutively expressed and not affected by Stat3-C (Fig. 5(D)).

3.6. Overexpression of Mn-SOD reduces HR-induced apoptosis

To examine whether Mn-SOD by itself exerts protective effects against H/R-induced apoptosis, Mn-SOD was adenovirally overexpressed in hepatocytes, which then underwent H/R insult. Mn-SOD was induced by AdMn-SOD transfection in hepatocytes (Fig. 6(A)), which significantly reduced H/R-induced apoptosis (Fig. 6(B)). Apoptotic cell death was significantly decreased by AdMn-SOD transfection, which became more prominent with an increase of the amounts of adenovirus transfected.

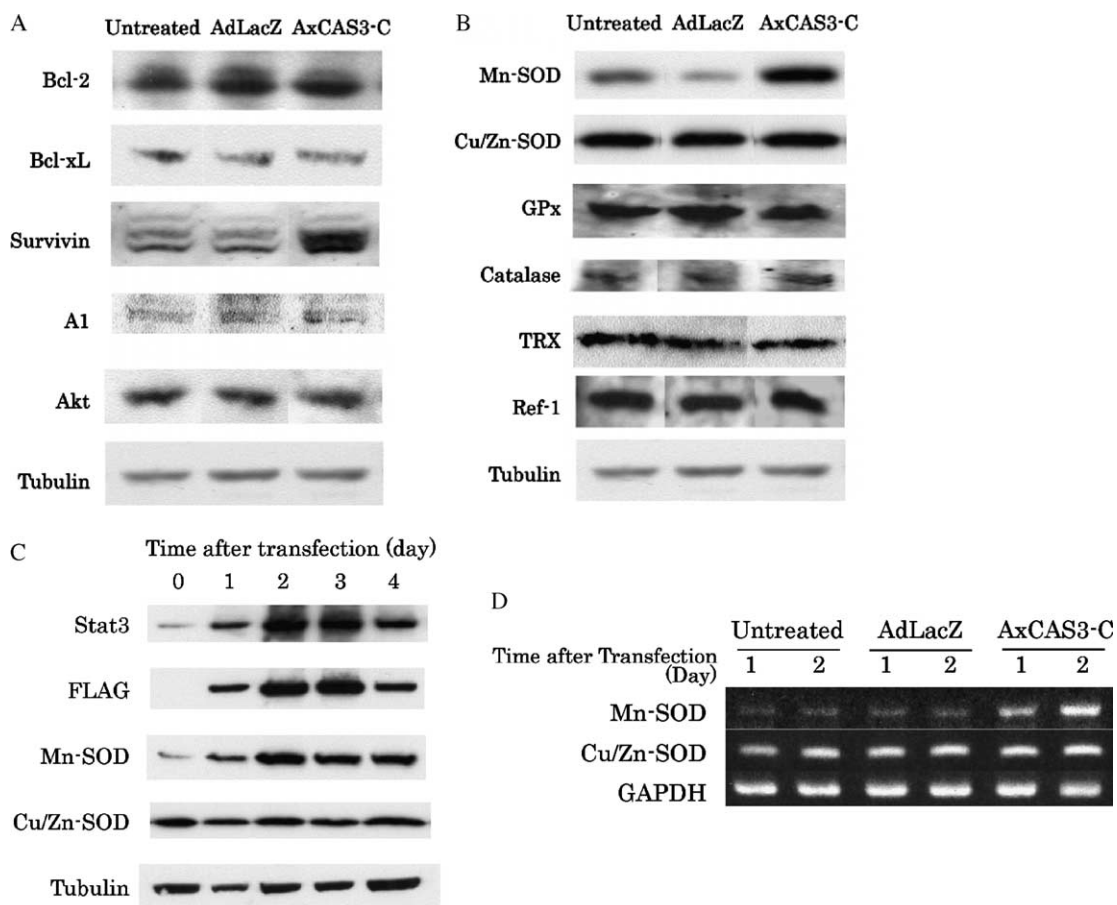


Fig. 5. Stat3-C-induced anti-apoptotic and anti-oxidant proteins. (A) Anti-apoptotic proteins induced by Stat3. (B) Anti-oxidant proteins induced by Stat3. (C) Increased protein level of Mn-SOD induced by Stat3. Lysates from hepatocytes transfected with AdLacZ or AxCAS3-C 2 days before (indicated time point in (C)) were analyzed by Western blotting. Survivin and Mn-SOD were time-dependently by Stat3 overexpression. (D) Induction of Mn-SOD transcription by Stat3. Total RNA was extracted 1 and 2 days after transfection with AdLacZ or AxCAS3-C and analyzed by RT-PCR. Mn-SOD, Cu/Zn-SOD and GAPDH primers were used. (E) Increase of Mn-SOD activity induced by Stat3-C. Lysates from hepatocytes transfected by AdLacZ or AxCAS3-C 2 days before were applied to the assay. Transcription of Mn-SOD but not Cu/Zn-SOD was increased by Stat3 overexpression. * $P < 0.005$ versus AdLacZ group.

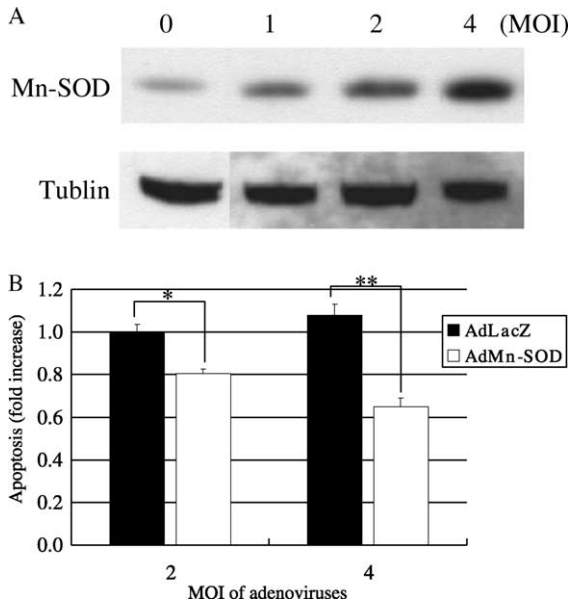


Fig. 6. Overexpression of Mn-SOD reduces H/R-induced apoptosis. (A) Overexpression of Mn-SOD by AdMn-SOD transfection. Lysates of hepatocytes were collected 2 days after transfection of AdMn-SOD at indicated MOI, and were analyzed by Western blotting. Mn-SOD was overexpressed by AdMn-SOD transfection dose-dependently. (B) Protective effect of Mn-SOD against H/R-induced apoptosis. AdLacZ or AdMn-SOD was transfected 2 days before hypoxia, and culture medium collected 8 h after reoxygenation. Longitudinal axis indicates increase of apoptotic level during 0–8 h after reoxygenation. Numeric values represent the fraction relative to the untreated group. Apoptosis 8 h after H/R was significantly decreased by Mn-SOD overexpression dose-dependently. * $P < 0.05$; ** $P < 0.005$.

3.7. Stat3-C inhibits H/R-induced redox-sensitive caspase activity

To investigate the mechanism by which suppression of ROS leads to inhibition of apoptosis, protein expression of caspases 4 h after reoxygenation was measured. Following H/R, caspase-3 was cleaved and activated (17/19 kDa) in the hepatocytes with no adenovirus and in those transfected with AdLacZ, whereas the signal disappeared in either AxCAS3-C or AdMn-SOD transfects, while caspase-9 remained unchanged (Fig. 7).

4. Discussion

This study revealed the protective effect of Stat3 against H/R-induced apoptosis in primary hepatocytes. Although the properties of Stat3 for cell protection have been studied previously, this is the first report of its protective effects on primary cultured hepatocytes in a pathological condition such as H/R. The underlying mechanism of this effect must involve at least upregulation of Mn-SOD, which is indeed upregulated by Stat3. Mn-SOD scavenges H/R-induced cellular ROS that may function as signaling intermediaries,

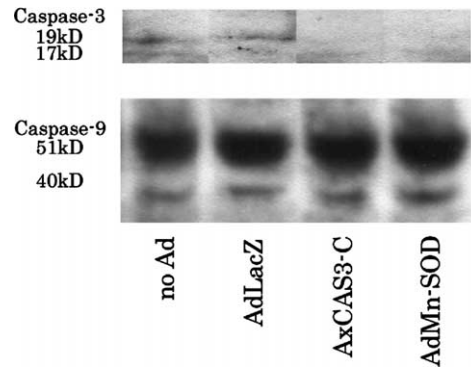


Fig. 7. Inhibition of H/R-induced caspase-3 activation by Stat3-C and Mn-SOD. Hepatocyte was transfected with AdLacZ, AxCAS3-C and AdMnSOD 2 days before H/R. Cell lysates were collected 4 h after H/R and were analyzed by Western blotting. Cleavage of caspase-3 (17/19 kDa) induced by H/R was decreased by overexpression of Stat3 or Mn-SOD.

and consequently inactivates redox-dependent, pro-apoptotic caspase-3.

In the present study, most of the hepatocytes underwent apoptosis 8 h after reoxygenation. IL-6 and CT-1 decreased the apoptotic cell death by 66 and 77% compared to the control, respectively. This observation is consistent with other reports of protective effects of IL-6 against I/R-induced liver injury [34,35] and anti-apoptotic effects of hepatic CT-1 [36]. These anti-apoptotic properties of IL-6 family proteins seem to result primarily from activating downstream signaling molecules including Jak/Stat, MAPK and phosphatidylinositol 3-kinase (PI3-K) [37], but it still remains unclear which pathways are most critically involved. Adenovirally overexpressed Stat3-C in hepatocytes decreased the apoptotic cell death by 57% compared to control vector. This anti-apoptotic effect is almost the same as that induced by IL-6, suggesting that the Jak/Stat pathway may play critical roles in anti-apoptotic effects of IL-6.

Though Jak/Stat pathway seems to be an essential and main pathway in IL-6-mediated Stat3 activation and Mn-SOD induction, Stat3 is also known sometimes to interact with other Stat proteins such as Stat1 and Stat6. Interestingly, Stat1 was significantly activated (phosphorylated at serine727 and tyrosine701) by Stat3-C overexpression without affecting the amount of Stat1 protein (Fig. S1). However, Stat6 is not phosphorylated at all by Stat3-C. Direct interaction of Stat3 with these proteins in inactive forms is still unknown, but it seems that activation of Stat1 is at least affected by Stat3.

H/R-induced apoptosis is considered to result mainly from ROS generation [3], and a number of reports showed that reduced ROS generation suppressed injury after reoxygenation by catalytic anti-oxidants [38,39] or anti-oxidative enzymes including catalase [40], GPx [41], Cu/Zn-SOD [42], Mn-SOD [10] and TRX [43]. In the present study, ROS generation after H/R was suppressed by Stat3 overexpression. This led us to examine the anti-oxidative

mechanisms in Stat3-induced cellular protection, and we showed transcriptional upregulation of Mn-SOD by Stat3 in hepatocytes. Interestingly, adenoviral overexpression of Mn-SOD inhibited both ROS generation and apoptotic cell death after reoxygenation to the same degree as Stat3 (Figs. 3(B), 4(C) and 6(B)). Upregulation of only Mn-SOD but not catalase and GPx seems to induce increase in H₂O₂. However, it is generally considered that catalase and GPx exists in cytoplasm broadly and abundantly, so Mn-SOD upregulation results in reduction of oxidative stresses. These findings imply that Stat3 confers resistance against H/R-induced oxidative injury to hepatocytes mainly through upregulation of Mn-SOD. This idea was supported by the previous *in vivo* I/R studies where administration or adenoviral overexpression of Mn-SOD reduced liver damage [10,44]. It has already been reported that Stat3-induced Mn-SOD protects cardiomyocytes from H/R stress [25]. Although that study did not refer to the mode of cell death (apoptosis or not) and why ROS generation decreased by Mn-SOD results in cellular protection, it supports our present data that Mn-SOD plays a crucial role in Stat3-induced anti-apoptotic effects. Considering that ROS-induced apoptosis is mediated by redox-dependent caspase-3 [45–47], the anti-apoptotic effect induced by Mn-SOD could result at least partially from reduced ROS generation and subsequent caspase-3 inactivation.

As possible mediators of its anti-apoptotic properties, it is known that Stat3 induces various molecules such as survivin [48–50], Bcl-2 [51,52] or Bcl-xL [50,52], in a cell-type and stress specific manners. In hepatocytes, we showed that survivin and Mn-SOD were upregulated by Stat3-C, while Bcl-2 and Bcl-xL remained stable. Considering the many functions of Stat3, it is still unclear how far the other anti-apoptotic molecules (e.g. survivin) are involved in anti-apoptotic effects on H/R-stressed hepatocytes.

In order to address the clinical relevance of Stat3 in the protection of hepatic I/R-injury, we have also introduced Stat3-C adenovirally to liver and tried to see its effect upon protection of post-ischemic liver (1-hr warm ischemia, 8-hr reperfusion). Unfortunately, we could not get sufficient data to convince clinical relevance of Stat3. Stat3-C improved liver injury compared with LacZ-mice, but not with uninfected control mice. The limit of this experiment is that we had to use adenoviral vector, which by itself must have injured liver (Fig. S2). Stat3-C seems to improve I/R-induced liver injury, but adenoviral gene transfer to the liver was more harmful to the liver.

We have demonstrated successful inhibition of H/R-induced apoptosis of hepatocytes by activation of Stat3 and investigated its underlying mechanisms. Though further studies are required, we conclude that Stat3 confers resistance against H/R-induced oxidative injury in hepatocytes through Mn-SOD. This may provide a new approach to cell protection as a molecular targeting therapy in different clinical situations.

Acknowledgements

We thank Dr James E. Darnell for kindly providing Stat3-C cDNA.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2004.08.019

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