Hypoxia/re-oxygenation-induced, redox-dependent activation of STAT1 (signal transducer and activator of transcription 1) confers resistance to apoptotic cell death via *hsp70* induction

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STAT1 (signal transducer and activator of transcription 1) is potentially involved in cell survival, as well as cell death, in different types of cells. The present study was designed to examine the effects of STAT1 on hypoxia/re-oxygenation (H/R)-induced cell death and/or survival, and the underlying mechanisms of any such effects. H/R was shown to induce apoptotic cell death of rat hepatocytes. The addition of a STAT1-specific inhibitor, fludarabine, significantly increased the fraction of apoptotic cells after H/R. Following H/R, STAT1 was activated and sequential phosphorylation of Tyr^{701} and Ser^{727} was observed, which could be inhibited by the antioxidant *N*-acetyl-L-cysteine. Tyrosine and serine phosphorylation of STAT1 was mediated by Janus kinase 2 and phosphoinositide 3-kinase/Akt kinase respectively in a redox-dependent manner following H/R. STAT1-induced HSP70 (heat-shock protein 70) expression and the suppression of apoptosis occurred concomitantly. In conclusion, STAT1 activation, in a redox-dependent manner, following H/R may play crucial roles in cell survival, at least partly via HSP70 induction.

Key words: apoptosis, heat-shock protein 70 (HSP70), hepatocyte, hypoxia/re-oxygenation, oxidative stress, signal transducer and activator of transcription 1 (STAT1).

INTRODUCTION

Hepatic ischaemia/reperfusion-induced injury is still a major concern in various clinical settings, involving liver transplantation and general liver surgery. The structure and function of liver, which has suffered ischaemic insult, is known to deteriorate during the post-ischaemic period. Several factors are thought to contribute to the pathogenesis of this post-ischaemic liver injury, with the generation of ROS (reactive oxygen species) possibly being one of the most important. Cellular ROS generated immediately after re-oxygenation will affect physiological cell signalling [1–5]. Such signals triggered by cellular ROS in some pathological situations may lead to liver injury through the activation of pro-apoptotic and pro-inflammatory genes [3,6]. However, such ROS may also contribute to the induction of certain molecules important in overcoming the insult and facilitating cell survival. HSP70 (heat-shock protein 70) is possibly one of these proteins, and is well known to be involved in cellular protection against ischaemic (hypoxic) insult [7–9]. This protein is induced by oxidative stress such as hypoxia/re-oxygenation (H/R), and may function as an anti-apoptotic molecule [10–14]. Superoxide dismutase is also induced by transient oxidative stress, and then protects against more prolonged oxidative stress by scavenging superoxide anions [15–19]. Additionally, through their surface receptors, different cytokines and growth factors activate intracellular signals involving JAKs (Janus kinases) and STATs (signal transducers and activators of transcription). The latter are phosphorylated on Tyr^{701}, dimerized and fully activated by phosphorylation on Ser^{727}, followed by translocation into the nucleus. Previous studies have implicated STATs in both pro- and anti-apoptotic signalling [20–23]. For example, a dominant negative form of STAT3 has been shown to prevent interleukin-6-dependent cell growth and to induce apoptosis in a myeloid cell line [24]. Reciprocally, a constitutively activated form of STAT3 protects against UV-irradiation- and serum-withdrawal-induced apoptosis in fibroblasts [25], and against Fas-mediated liver injury [26]. In contrast, a STAT1-deficient cell line is resistant to tumour necrosis factor α-induced apoptosis [27], and STAT1 has been implicated in transcriptional activation of caspases in fibroblasts [28]. STAT1 is phosphorylated and activated significantly during cardiac ischaemia without reperfusion, and is suggested potentially to play a critical role in the apoptotic cell death of cardiomyocytes after reperfusion [21,22]. Ischaemia-induced caspase activation mediated by STAT1 may account for the observed post-ischaemic apoptotic cell death, at least in the heart. Oxidative stress, however, up-regulates HSP70 via the JAK–STAT pathway in vascular smooth muscle cells [23]. It has also been suggested that STAT1 activated by IFN (interferon)-γ interacts with HSF-1 (heat-shock fatcor-1) to induce HSP70/HSP90β in HepG2 cells [20]. These findings suggest that STAT1 possesses both pro- and anti-apoptotic properties which are activated in response to some unique physiological/pathological stimuli, though the mechanistic basis for these effects is still unclear.

The present study was designed to examine the mechanism of H/R-induced STAT1 activation and the effects of STAT1 on primary cultured rat hepatocytes which have undergone H/R. After re-oxygenation, activated STAT1 demonstrated pro-survival (anti-apoptotic) properties, which was concomitant with the induction of HSP70. Thus the protective effect of STAT1 against H/R insult may be explained at least partly by HSP70 induction.

Abbreviations used: EMSA, electrophoretic mobility-shift assay; GSK-3, glycogen synthase kinase-3; H/R, hypoxia/re-oxygenation; HSF-1, heat-shock factor-1; HSP, heat-shock protein; IFN, interferon; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetyl-L-cysteine; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription.

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EXPERIMENTAL

Reagents

IFN-γ and recombinant human interleukin-6 were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Chemical inhibitors including wortmannin, Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate]), PD98059, SP600125 and SB220025 were obtained from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical grade and were used without further purification.

Primary cultured rat hepatocytes and H/R procedures

Primary hepatocytes from rat liver (Lewis rats, 250 g, male) were prepared by the conventional perfusion method with collagenase digestion. Rat hepatocytes were seeded at 3×10^6 cells per dish in 10 cm diameter plastic dishes coated with rat tail collagen and cultured in William's E complete medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10 % fetal bovine serum, 1 nmol/l insulin and 1 nmol/l dexamethasone in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C. The fibroblast cell lines, 2ftgh and its STAT1-deficient variant U3A, were kindly provided by Dr Ian M. Kerr (Cancer Research UK London Research Institute, London, U.K.). In order to simulate an ischaemic liver *in vitro*, hypoxic condition was maintained in a modulator incubator chamber (Billups-Rothenberg, Del Mar, CA, U.S.A.) by flushing with a 95 % N_2/5 % CO_2 gas mixture for 10 min and then sealing the chamber. This method has been shown to achieve a pO_2 of 10 ± 5 Torr (where 1 Torr = 0.133 kPa). Following 4 h of hypoxia, re-oxygenation of hepatocytes was achieved by opening the chamber and replacing the hypoxic medium with oxygenated medium. The antioxidant NAC (*N*-acetyl-L-cysteine) and drugs such as fludarabine, AG490, MAPK (mitogen-activated protein kinase) inhibitors or PI3K (phosphoinositide 3-kinase)/Akt inhibitors were added 2 h prior to the hypoxic insult. All animals were handled according to uniform policies set forth by the Animal Care and Use Committee of National Research Institute for Child Health and Development.

EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared as described previously [29]. Nuclear extract (5 μg) was incubated with 10^6 c.p.m. of an end-labelled consensus sequence oligonucleotide of STAT1 (5′-CATGTTATGCATATTCCTGTAAGT-3′, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or mutant oligonucleotide of STAT1 (5′-CATGTTATGCATATTGGAGTAAGT-3′, Santa Cruz Biotechnology) for 30 min at 25 °C in binding buffer (10 mM Tris, pH 7.4, 80 mM KCl, 5 % glycerol, 0.5 mM dithiothreitol, 2 μg of dIdC, 10 μg of BSA). Protein–DNA complexes were resolved on a 4 % polyacrylamide gel and subjected to autoradiography.

Apoptosis assay

For evaluation of apoptosis, an ELISA kit (Cell Death Detection ELISAPLUS; Roche, Basel, Switzerland) was used according to the manufacturer's instructions. This test is based on the detection of mono- and oligo-nucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated anti-histone antibodies and peroxidase-coupled anti-DNA antibodies. Aliquots of the culture media at the time points indicated were used for the apoptosis assay. Double staining of hepatocytes with Hoechst33342 (Hoechst) and propidium iodide (PI) was also employed to confirm apoptotic hepatocytes. Hepatocytes were stained with Hoechst (10 μM) and PI (10 μM) 5 min prior to fluorescence microscopic observation. Hepatocytes whose nuclei showed fragmented DNA, stained in blue with Hoechst, with no red stain (PI) were considered as apoptotic hepatocytes, because PI can permeate cell membrane of only necrotic cells. For each dish, 10 randomly selected microscopic fields were recorded and the number of apoptotic cells was counted at the time points indicated.

Assays of STAT1 phosphorylation and Akt activity

Whole cell extracts (20 μg) were resolved by SDS/PAGE on a 10 % gel and transferred on to a nitrocellulose membrane. Total and phosphorylated STAT1 were detected using anti-STAT1 antibody and anti-(phospho-STAT1) antibody (phosphorylated at Tyr^{701} and Ser^{727}; Upstate biotechnology, Lake Placid, NY, U.S.A.). The Akt activity assay was performed using Akt Kinase Assay Kit (Cell Signaling, Beverly MA, U.S.A.). Whole cell extracts from 3×10^6 hepatocytes were immunoprecipitated with immobilized anti-Akt antibody, and *in vitro* kinase assays were performed using GSK (glycogen synthase kinase-3)-fusion protein as a substrate according to the manufacturer's instructions. Phosphorylation of GSK-3 was detected with anti-(phospho-GSK-3).

Northern blot analysis

After electrophoresis on a 1.4 % agarose gel, total cellular RNA was transferred on to a nylon membrane, which was hybridized with 3×10^7 c.p.m. of ^{32}P-labelled single-strand cDNA of human inducible *HSP70*. After washing under stringent conditions, the membrane was subjected to autoradiography. The membrane was then stripped and rehybridized with a probe to 18 S rRNA and autoradiographed.

Statistical analysis

All results are expressed as the means $\pm$ S.E.M. for at least three independent experiments. A one-way ANOVA was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

STAT1 is involved in the survival of hepatocytes following H/R

We first examined whether STAT1 is involved in H/R-induced cell apoptosis in primary cultured hepatocytes. A period of 4 h of hypoxia without re-oxygenation was not sufficient to induce significant apoptosis in untreated hepatocytes. Apoptotic cell death caused by H/R, however, was maximal 8 h after re-oxygenation, without any further increase observed over a period of up to 72 h. To examine the involvement of STAT1 in this H/R-induced apoptosis, hepatocytes were pretreated with a specific STAT1-inhibitor, fludarabine [30,31]. Although the precise mechanism and specificity of fludarabine-mediated STAT1 inhibition remains unclear, fludarabine is the only available inhibitor of STAT1 which was proven to have specificity, at least, for the JAK–STAT signalling pathway [31] and does not inhibit STAT3 activity (results not shown). Pre-treatment with this drug caused re-oxygenated hepatocytes to continue to be susceptible to apoptotic cell death for up to 72 h after re-oxygenation (Figure 1a). H/R-induced apoptotic cell death and the effect of fludarabine were confirmed morphologically by another method using PI and Hoechst stains, which showed the same results as Figure 1a (Figure 1b). Although fludarabine contributed to the cell death

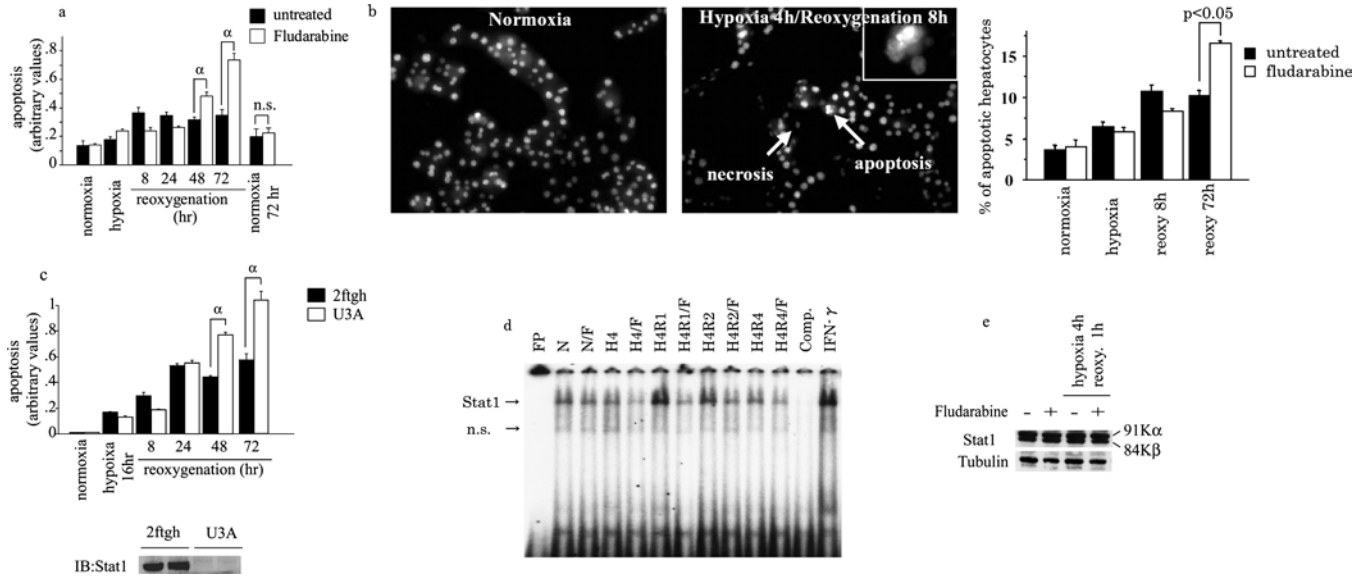

Figure 1 STAT1 is involved in cell survival following H/R in primary hepatocytes

(**a**) ELISA assay for H/R-induced apoptotic cell death in hepatocytes. Fludarabine (50 μM), a specific STAT1 inhibitor, was added 2 h prior to the H/R experiment. Aliquots of the culture media at the time points indicated were used for the apoptosis assay. α, $P > 0.05$; n.s., not significant. The results are expressed as the means $\pm$ S.E.M. (**b**) Fluorescence micrograph of cultured primary hepatocytes after H/R stained with Hoechst and PI. (original magnification, $\times$ 100). Hepatocytes were stained with Hoechst (10 μM) and PI (10 μM) 5 min prior to fluorescence microscopic observation. Hepatocytes whose nuclei show fragmented DNA, stained in blue with Hoechst, with no red stain (PI) are considered as apoptotic hepatocytes. For each dish, 10 randomly selected microscopic fields were recorded and the number of apoptotic cells was counted at the time points indicated. (**c**) ELISA assay for H/R-induced apoptotic cell death of 2ftgh and U3A cells. Aliquots of the culture media at the time points indicated were used for the apoptosis assay. No expression of STAT1 protein in U3A cells was confirmed by Western blot analysis. α, $P < 0.05$. The results are expressed as the means $\pm$ S.E.M. (**d**) EMSA for STAT1 DNA binding after H/R. Nuclear extract (5 μg) was incubated with 10^6 c.p.m. of an end-labelled consensus sequence oligonucleotide of STAT1 for 30 min at 25 °C in binding buffer. Protein–DNA complexes were resolved on a 4 % polyacrylamide gel and subjected to autoradiography. FP, free probe; N, normoxia; H4, hypoxia 4 h; H4R1, hypoxia 4 h + re-oxygenation 1 h; F, fludarabine; Comp, competition ($\times$ 200). (**e**) STAT1 protein in the environment with or without fludarabine. Fludarabine (50 μM) was added 2 h prior to the experiment. Whole cell extracts (20 μg) were applied to Western blot analysis using anti-STAT1 antibody. STAT1α, 91 kDa (91Kα); STAT1β, 84 kDa (84Kβ). The results in (**d**) and (**e**) are representative of at least 3 independent experiments.

after H/R, it did not affect the viability of hepatocytes without H/R until 72 h after addition (Figure 1a).

In order to confirm this pro-survival effect of STAT1 on H/R-induced cell death, we also performed the same experiment using a fibroblast cell line expressing STAT1 (2ftgh) and a STAT1-deficient variant of this line (U3A) [27]. Up to 24 h after H/R, the number of apoptotic cells in both the 2ftgh and U3A lines increased. However, U3A cells showed further increases of apoptotic cell death 48–72 h after H/R, whereas 2ftgh did not (Figure 1c).

H/R-induced STAT1 activation was measured by its DNA-binding capability. STAT1 was mildly and weakly activated even under normoxic and hypoxic states. However, its DNA binding activity was remarkably increased after re-oxygenation, which was effectively inhibited by pre-treatment of fludarabine with no change in the amount of cellular STAT1 protein (Figures 1d and 1e). Fludarabine inhibited STAT1 activation, even in normoxic and hypoxic states.

H/R phosphorylates STAT1 on Tyr^{701} and Ser^{727} sequentially through JAK2 and PI3K/Akt in a redox-dependent manner

To understand the mechanism of STAT1 activation by H/R, we next investigated phosphorylation of STAT1 following H/R. Generally, STAT1 is sequentially phosphorylated on Tyr^{701} and Ser^{727} following ligand-mediated stimulation. Tyrosine phosphorylation is essential for any STAT1 activation, and serine phosphorylation is additionally required for full activation [32].

Immediately following H/R, STAT1 was transiently phosphorylated on Tyr^{701}, and then on Ser^{727} (Figure 2a). Fludarabine inhibited H/R-induced phosphorylation of STAT1 both on Tyr^{701} and Ser^{727}. Although STAT1 was weakly, but constitutively, phosphorylated on Tyr^{701}, pre-treatment of hepatocytes with the Jak2 inhibitor AG-490 significantly inhibited H/R-induced STAT1 phosphorylation on Tyr^{701} (Figure 2b). Unexpectedly, Ser^{727} phosphorylation, however, was not inhibited by pre-treatment with MAPK inhibitors, PD98059, SP600125 or SB220025 (Figure 2c). In contrast, the PI3K inhibitor, wortmannin, and the Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol 2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate], dramatically inhibited STAT1 phosphorylation on Ser^{727} (Figure 2d). Interestingly, the STAT1 inhibitor, fludarabine, and antioxidant, NAC, inhibited H/R-induced phosphorylation of STAT1 both on Tyr^{701} and Ser^{727}, although they failed to inhibit tyrosine phosphorylation completely.

As it was unexpectedly shown that Akt is involved in STAT1 phosphorylation on Ser^{727}during H/R, we also examined phosphorylation and activation of Akt by H/R and an oxidant (H_2O_2) (Figure 3). Akt was phosphorylated (weakly on Thr^{308} and strongly on Ser^{473}) and activated immediately after H/R, and this was maintained for at least 120 min (Figure 3a). H_2O_2 and H/R-induced Akt activation was partially, but significantly, inhibited by pre-treatment with NAC or catalase, as expected (Figure 3b). These findings may imply that oxidative stress activates two types of kinases, JAK2 and PI3K/Akt, which eventually result in STAT1 activation by phosphorylation of Tyr^{701} and Ser^{727} respectively.

H/R induces STAT1 DNA binding via oxidative stress

Next, we examined whether STAT1 DNA binding induced by H/R is mediated by oxidative stress. We first investigated whether oxidants may directly increase STAT1 DNA binding (Figure 4a).

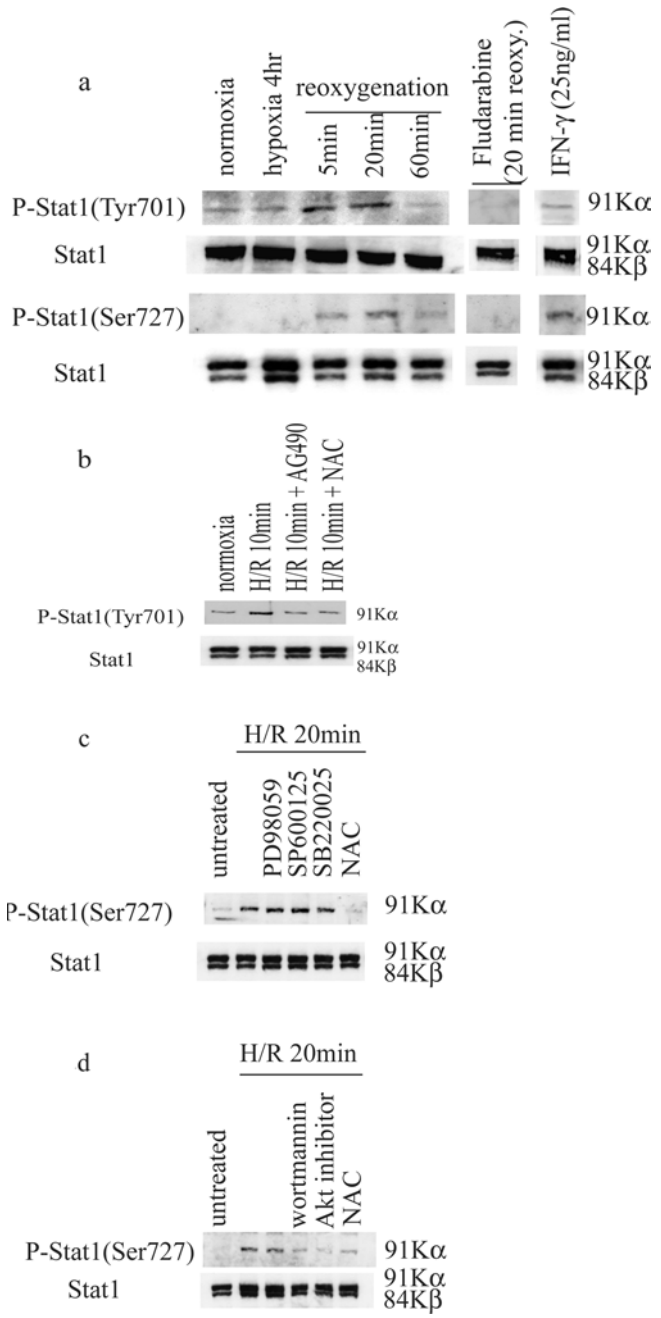

Figure 2 H/R phosphorylates STAT1 through Jak2 and PI3-K/Akt in a redox-dependent manner

(**a**) H/R-induced STAT1 phosphorylation on Tyr^{701} and Ser^{727} and their inhibition by fludarabine. Fludarabine was added 2 h prior to the H/R experiment. Whole cell extracts at the time points indicated were subjected to Western blot analysis using anti-(phospho-STAT1) (P-STAT1) antibody. (**b**) Inhibition of H/R-induced STAT1 phosphorylation on Tyr^{701} by the Jak2 inhibitor, AG490. AG490 (50 μM) or the antioxidant NAC (1 mM) was added to the culture medium 2 h prior to H/R. Whole cell extracts were subjected to Western blot analysis. (**c**) Effects of MAPK inhibitors on STAT1 phosphorylation on Ser^{727}. MAPK inhibitors (PD98059, 25 μM; SP600125, 40 nM; SB220025, 60 nM) were added to the culture medium 2 h prior to H/R. Whole cell extracts were subjected to Western blot analysis. (**d**) Effects of PI3K/Akt inhibitors and NAC on STAT1 phosphorylation on Ser^{727}. PI3K inhibitors (wortmannin, 2 μM), Akt inhibitors (1 μM) or NAC was added to the culture medium 2 h prior to H/R. Whole cell extracts were subjected to Western blot analysis. The results are representative of at least three independent experiments.

The DNA binding was weakly activated, even in untreated cells, and increased by the addition of H_2O_2 (5–1000 μM), showing maximal activity at 300 μM. Hydroxyl radicals generated from

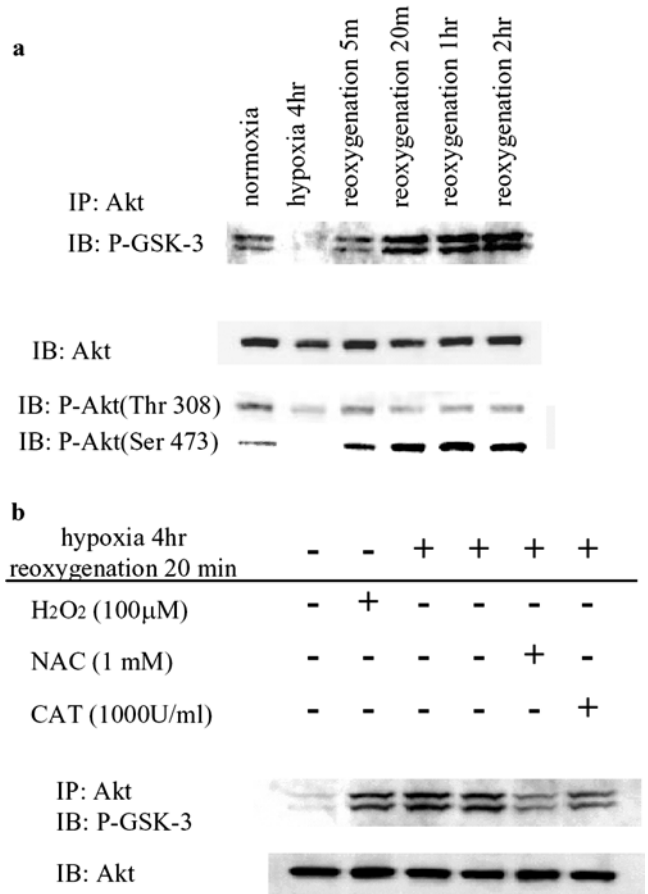

Figure 3 Akt is phosphorylated and activated by H/R in a redox-dependent manner

(**a**) Phosphorylation and activation of Akt in primary hepatocytes following H/R. Whole cell extracts, at the time points indicated, were immunoprecipitated (IP) with immobilized α-Akt and subjected to kinase assays using GSK-3-fusion protein as a substrate, or to Western blot analysis using anti-Akt and anti-(phospho-Akt) (Thr^{308} and Ser^{473}) antibodies. (**b**) Inhibition of an oxidant and H/R-induced Akt activation by antioxidants. H_2O_2, NAC and catalase (CAT) were added to the culture medium 2 h prior to H/R and whole cell extracts were subjected to Akt kinase assays or Western blot analysis. The results are representative of at least three independent experiments.

H_2O_2 by reaction with $FeSO_4$ and $CuSO_4$ (100 μM of each) stimulated DNA binding in the range 30–125 μM H_2O_2, with the signal peaking at 125 μM (results not shown) [33]. Because hydroxyl radicals are more reactive than H_2O_2, STAT1 activation may have been stimulated at lower concentrations compared with H_2O_2 alone. Furthermore, H/R-induced STAT1 DNA binding was significantly inhibited by pre-treatment with NAC (Figure 4b).

These results may indicate that STAT1 DNA binding is activated in a redox-dependent manner and induced uniquely and primarily by oxidative stress, including H/R.

H/R induces transcription and protein expression of HSP70

H/R up-regulated *HSP7O* transcription in hepatocytes 2 h after re-oxygenation, which was significantly reduced by pre-treatment with fludarabine (Figure 5a). Protein expression of HSP70 was also up-regulated 16–36 h after re-oxygenation (Figure 5b). The time interval between up-regulation of mRNA (2 h) and expression of protein (16 h) can be explained by considering the process of translation and protein synthesis. The anti-apoptotic effect

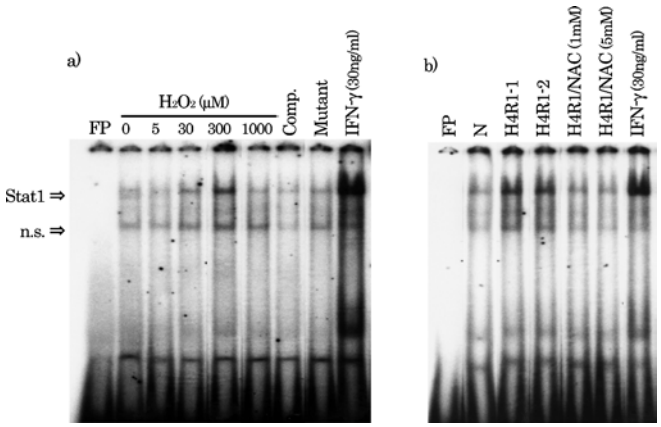

Figure 4 H/R and oxidative stress induce STAT1 DNA binding

(**a**) Induction of STAT1-DNA binding by oxidant stress (H_2O_2). H_2O_2 or IFN-γ were added to the culture medium 2 h prior to H/R. Nuclear extract (5 μg) was incubated with 10^6 c.p.m. of an end-labelled consensus sequence oligonucleotide of STAT1 for 30 min at 25 °C in binding buffer. Protein–DNA complexes were resolved on a 4 % polyacrylamide gel and subjected to autoradiography. (**b**) Inhibition of H/R-induced STAT1 DNA binding by antioxidants. NAC and IFN-γ were added 2 h prior to the hypoxic insult, and the nuclear extract was subjected to EMSA. FP, free probe; N, normoxia; Comp., competition ($\times$ 200); H4R1, hypoxia 4 h + re-oxygenation 1 h. The results are representative of at least three independent experiments.

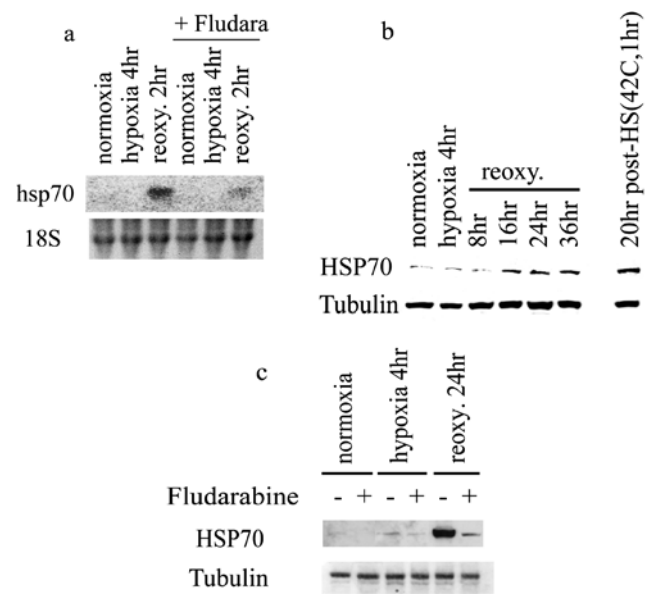

Figure 5 STAT1 up-regulates *HSP70* transcription and protein expression

(**a**) Inhibition of H/R-induced transcription of *HSP70* mRNA by the STAT1 inhibitor fludarabine. Fludarabine (50 μM) was added 2 h prior to the H/R. Total cellular RNA was separated by electrophoresis on a 1.4 % agarose gel, and was then transferred on to a nylon membrane and was hybridized with ^{32}P-labelled single-strand cDNA of human inducible *HSP70*. (**b**) HSP70 protein expression following H/R. Whole cell extracts at the time points indicated were subjected to Western blot analysis using anti-HSP70 antibody. HS; heat stress (42 °C for 1 h). (**c**) Inhibition of H/R-induced HSP70 protein expression by fludarabine. Fludarabine was added 2 h prior to the H/R and whole cell extracts were subjected to Western blot analysis. The results are representative of at least three independent experiments.

of STAT1 observed after 24 h of re-oxygenation may be explained by the late expression of HSP70 after H/R and its cytoprotective effects [11,13,34–36]. HSP70 protein expression was greatly reduced by pretreatment with the STAT1 inhibitor fludarabine (Figure 5c). This suggests that STAT1 is deeply involved in H/R-induced HSP70 induction and cell survival.

DISCUSSION

In the present report, it was first shown that H/R-induced, redox-dependent STAT1 activation is involved in cell survival at least partly via HSP70 induction mediated by STAT1. STAT1 does not seem to function in the early phase of re-oxygenation, but eventually acts as a pro-survival (anti-apoptotic) molecule in the late phase of re-oxygenation. Although the potential pro-apoptotic effects of STAT1 have already been reported [25], significant pro-survival (anti-apoptotic) effects were noted in the present study. Here, the pro-apoptotic effect of STAT1 may be explained by the fact that the STAT1 inhibitor fludarabine reduced the number of apoptotic cells 8 h following re-oxygenation (Figures 1a–1c).

STAT1 DNA binding could not be activated by hypoxia alone, but only by H/R in rat primary cultured hepatocytes. This is in marked contrast with rat heart, where STAT1 DNA binding was significantly activated by ischaemia alone [21,22]. The phosphorylation of STAT1 on both Tyr^{701} and Ser^{727} is generally required to obtain full activation by ligand-mediated stimuli. Although STAT1 may not have been stimulated by the specific ligand here, it was phosphorylated and activated after H/R in exactly the same manner. However, the redox-dependent mechanism seems to underlie STAT1 activation by H/R. Following H/R, STAT1 phosphorylation on Tyr^{701} was considered to be mediated by JAK2, because AG490, a JAK2 inhibitor, reduced it to the basal level. The antioxidant, NAC, showed the same effects as AG490, which may indicate that Jak2 is also regulated by oxidative stress [37–39]. Unexpectedly, Ser^{727} of STAT1 was found to be phosphorylated by PI3K/Akt, not by MAPK. Although PI3K/Akt and MAPK are definitely activated by oxidative stresses [40–43], only the PI3K–Akt pathway was involved in phosphorylating STAT1 on Ser^{727} on H/R. In support of this, Akt, a serine/threonine kinase, was activated immediately after H/R with phosphorylation on Thr^{308} and Ser^{473}, which was prevented by pre-treatment with the antioxidants NAC and catalase. Taken together, these results suggest that a redox-dependent system is the underlying mechanism of H/R-induced STAT1 activation.

STAT1 is known to target some pro-apoptotic genes, such as caspase-1, Cpp32 (cysteine protease protein of mass 32 kDa), Ich-1 (also named as caspase-2) and Fas [21,22,27], but, on the other hand, it is also involved in HSP70/HSP90β induction in vascular smooth muscle cells and HepG2 cells [20,23]. Interestingly, HSP70/HSP90β induction in HepG2 cells can result from the interaction of STAT1 with HSF-1 [20]. Therefore, it appears that STAT1 possesses potent dual and, seemingly, opposing functions, being both pro- and anti-apoptotic (pro-survival), and is regulated by distinct mechanisms in response to specific stimuli. In the present study, the STAT1 inhibitor fludarabine dramatically increased apoptotic cell death 48–72 h after re-oxygenation, whereas apoptosis was decreased within 24 h of re-oxygenation. This finding was confirmed in a different model, because it has not been completely proved if fludarabine is specific for STAT1 inhibition. Response to the H/R insult of STAT1-deficient variant (U3A) and control (2ftgh) fibroblast cell lines were observed to confirm the results obtained with hepatocytes. STAT1 inhibition functions as pro-apoptotic in the early phase of re-oxygenation and as anti-apoptotic in the late phase. The precise mechanism of this opposite response of hepatocytes to fludarabine is unclear, but it may be speculated as follows. The pro-apoptotic effect of STAT1 appears quickly, because it mainly results from post-translational modifications, including cleavage of caspases. Therefore in the early phase, the prompt pro-apoptotic response comes to the foreground more powerfully. After that, the pro-apoptotic effect of STAT1 seems to be attenuated, which is supported by the results that the peak of the caspase-3 cleavage was observed approx. 8 h after

re-oxygenation, and it is attenuated after 24 h (results not shown). Meanwhile, the protein level of HSP70 was increased 16 h after re-oxygenation. This anti-apoptotic effect is brought to the surface as the pro-apoptotic effect is attenuated in the late phase. Although HSP70 protein was induced by STAT1, it still remains unclear whether it is necessary or sufficient for conferring resistance against apoptosis.

The precise mechanisms by which STAT1 promotes survival are still unclear. STAT1 is undoubtedly activated in a redox-dependent manner following H/R, and is involved in cell survival. STAT1, however, may also be involved in various unique functions in response to specific stimuli. Although further studies are clearly required, the present investigation provides some insight for understanding the redox-dependent regulation of STAT1 and its functions.

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REFERENCES

1 Ng, C. K., Deshpande, S. S., Irani, K. and Alevriadou, B. R. (2002) Adhesion of flowing monocytes to hypoxia-reoxygenation-exposed endothelial cells: role of Rac1, ROS, and VCAM-1. Am. J. Physiol. Cell Physiol. **283**, C93–C102

2 Nardo, B., Caraceni, P., Pasini, P., Domenicali, M., Catena, F., Cavallari, G., Santoni, B., Maiolini, E., Grattagliano, I., Vendemiale, G. et al. (2001) Increased generation of reactive oxygen species in isolated rat fatty liver during postischemic reoxygenation. Transplantation **71**, 1816–1820

3 Ozaki, M., Suzuki, S. and Irani, K. (2002) Redox factor-1/APE suppresses oxidative stress by inhibiting the rac1 GTPase. FASEB J. **16**, 889–890

4 Kunduzova, O. R., Bianchi, P., Pizzinat, N., Escourrou, G., Seguelas, M. H., Parini, A. and Cambon, C. (2002) Regulation of JNK/ERK activation, cell apoptosis, and tissue regeneration by monoamine oxidases after renal ischemia-reperfusion. FASEB J. **16**, 1129–1131

5 Chien, C. T., Lee, P. H., Chen, C. F., Ma, M. C., Lai, M. K. and Hsu, S. M. (2001) *De novo* demonstration and co-localization of free-radical production and apoptosis formation in rat kidney subjected to ischemia/reperfusion. J. Am. Soc. Nephrol. **12**, 973–982

6 Ozaki, M., Deshpande, S. S., Angkeow, P., Bellan, J., Lowenstein, C. J., Dinauer, M. C., Goldschmidt-Clermont, P. J. and Irani, K. (2000) Inhibition of the Rac1 GTPase protects against nonlethal ischemia/reperfusion-induced necrosis and apoptosis *in vivo*. FASEB J. **14**, 418–429

7 Chong, K. Y., Lai, C. C., Lille, S., Chang, C. and Su, C. Y. (1998) Stable overexpression of the constitutive form of heat shock protein 70 confers oxidative protection. J. Mol. Cell Cardiol. **30**, 599–608

8 Suzuki, K., Sawa, Y., Kaneda, Y., Ichikawa, H., Shirakura, R. and Matsuda, H. (1998) Overexpressed heat shock protein 70 attenuates hypoxic injury in coronary endothelial cells. J. Mol. Cell Cardiol. **30**, 1129–1136

9 Dillmann, W. H. (1999) Heat shock proteins and protection against ischemic injury. Infect. Dis. Obstet. Gynecol. **7**, 55–57

10 Park, H. S., Cho, S. G., Kim, C. K., Hwang, H. S., Noh, K. T., Kim, M. S., Huh, S. H., Kim, M. J., Ryoo, K., Kim, E. K. et al. (2002) Heat shock protein Hsp72 is a negative regulator of apoptosis signal-regulating kinase 1. Mol. Cell. Biol. **22**, 7721–7730

11 Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M. and Green, D. R. (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat. Cell Biol. **2**, 469–475

12 Garrido, C., Gurbuxani, S., Ravagnan, L. and Kroemer, G. (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem. Biophys. Res. Commun. **286**, 433–442

13 Ravagnan, L., Gurbuxani, S., Susin, S. A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jaattela, M., Penninger, J. M., Garrido, C. and Kroemer, G. (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat. Cell Biol. **3**, 839–843

14 Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D. and Alnemri, E. S. (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat. Cell Biol. **2**, 476–483

15 Beresewicz, A., Czarnowska, E. and Maczewski, M. (1998) Ischemic preconditioning and superoxide dismutase protect against endothelial dysfunction and endothelium glycocalyx disruption in the postischemic guinea-pig hearts. Mol. Cell. Biochem. **186**, 87–97

16 Hoshida, S., Yamashita, N., Otsu, K. and Hori, M. (2002) The importance of manganese superoxide dismutase in delayed preconditioning: involvement of reactive oxygen species and cytokines. Cardiovasc. Res. **55**, 495–505

17 Suzuki, M., Takeuchi, H., Kakita, T., Unno, M., Katayose, Y. and Matsuno, S. (2000) The involvement of the intracellular superoxide production system in hepatic ischemia-reperfusion injury: *in vivo* and *in vitro* experiments using transgenic mice manifesting excessive CuZn-SOD activity. Free Radical Biol. Med. **29**, 756–763

18 Horie, Y., Wolf, R., Flores, S. C., McCord, J. M., Epstein, C. J. and Granger, D. N. (1998) Transgenic mice with increased copper/zinc-superoxide dismutase activity are resistant to hepatic leukostasis and capillary no-reflow after gut ischemia/reperfusion. Circ. Res. **83**, 691–696

19 Zwacka, R. M., Zhou, W., Zhang, Y., Darby, C. J., Dudus, L., Halldorson, J., Oberley, L. and Engelhardt, J. F. (1998) Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-kappaB activation. Nat. Med. **4**, 698–704

20 Stephanou, A., Isenberg, D. A., Nakajima, K. and Latchman, D. S. (1999) Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp-70 and Hsp-90β gene promoters. J. Biol. Chem. **274**, 1723–1728

21 Stephanou, A., Brar, B. K., Scarabelli, T. M., Jonassen, A. K., Yellon, D. M., Marber, M. S., Knight, R. A. and Latchman, D. S. (2000) Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. J. Biol. Chem. **275**, 10002–10008

22 Stephanou, A., Scarabelli, T. M., Brar, B. K., Nakanishi, Y., Matsumura, M., Knight, R. A. and Latchman, D. S. (2001) Induction of apoptosis and Fas receptor/Fas ligand expression by ischemia/reperfusion in cardiac myocytes requires serine-727 of the STAT-1 transcription factor but not tyrosine-701. J. Biol. Chem. **276**, 28340–28347

23 Madamanchi, N. R., Li, S., Patterson, C. and Runge, M. S. (2001) Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. Arterioscler. Thromb. Vasc. Biol. **21**, 321–326

24 Minami, M., Inoue, M., Wei, S., Takeda, K., Matsumoto, M., Kishimoto, T. and Akira, S. (1996) STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line. Proc. Nat. Acad. Soc. U.S.A. **93**, 3963–3966

25 Shen, Y., Devgan, G., Darnell, J. E. and Bromberg, J. F. (2001) Constitutively activated Stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated Stat1. Proc. Nat. Acad. Soc. U.S.A. **98**, 1543–1548

26 Haga, S., Terui, K., Zhang, H. Q., Enosawa, S., Ogawa, W., Inoue, H., Okuyama, T., Takeda, K., Akira, S., Ogino, T. et al. (2003) Stat3 protects against Fas-induced liver injury by redox-dependent and -independent mechanisms. J. Clin. Invest. **112**, 989–998

27 Kumar, A., Commane, M., Flickinger, T. W., Horvath, C. M. and Stark, G. R. (1997) Defective TNF-α-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science (Washington, D.C.) **278**, 1630–1632

28 Chin, Y., Kitagawa, M., Kuida, K., Flavell, R. and Fu, X. (1997) Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Mol. Cell. Biol. **17**, 5328–5337

29 Johnston, J. B., Jiang, Y., van Marle, G., Mayne, M. B., Ni, W., Holden, J., McArthur, J. C. and Power, C. (2000) Lentivirus infection in the brain induces matrix metalloproteinase expression: role of envelope diversity. J. Virol. **74**, 7211–7220

30 Frank, D. A., Mahajan, S. and Ritz, J. (1999) Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nat. Med. **5**, 444–447

31 Darnell, J. E. (1997) STATs and gene regulation. Science (Washington, D.C.) **277**, 1630–1635

32 Carballo, M., Conde, M., El Bekay, R., Martin-Nieto, J., Camacho, M. J., Monteseirin, J., Conde, J., Bedoya, F. J. and Sobrino, F. (1999) Oxidative stress triggers STAT3 tyrosine phosphorylation and nuclear translocation in human lymphocytes. J. Biol. Chem. **274**, 17580–17586

33 Creagh, E. M., Carmody, R. J. and Cotter, T. G. (2000) Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T-cells. Exp. Cell Res. **257**, 58–66

34 Suzuki, K., Sawa, Y., Kagisaki, K., Taketani, S., Ichikawa, H., Kaneda, Y. and Matsuda, H. (2000) Reduction in myocardial apoptosis associated with overexpression of heat shock protein 70. Basic Res. Cardiol. **95**, 397–403

35 Klein, S. D. and Brune, B. (2002) Heat-shock protein 70 attenuates nitric oxide-induced apoptosis in RAW macrophages by preventing cytochrome *c* release. Biochem. J. **362**, 635–641

36 Maziere, C., Conte, M. A. and Maziere, J. C. (2001) Activation of JAK2 by the oxidative stress generated with oxidized low-density lipoprotein. Free Radical Biol. Med. **31**, 1334–1340

37 Abe, J. and Berk, B. C. (1999) Fyn and JAK2 mediate Ras activation by reactive oxygen species. J. Biol. Chem. **274**, 21003–21010
38 Simon, A. R., Rai,U., Fanburg, B. L. and Cochran, B. H. (1998) Activation of the JAK-STAT pathway by reactive oxygen species. Am. J. Physiol. **275**, C1640–C1652
39 Wang, X., McCullough, K. D., Franke, T. F. and Holbrook, N. J. (2000) Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. J. Biol. Chem. **275**, 14624–14631
40 Ushio-Fukai, M., Alexander, R. W., Akers, M., Yin, Q., Fujio, Y., Walsh, K. and Griendling, K. K. (1999) Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J. Biol. Chem. **274**, 22699–22704
41 Haddad, J. J. and Land, S. C. (2002) Redox/ROS regulation of lipopolysaccharide-induced mitogen-activated protein kinase (MAPK) activation and MAPK-mediated TNF-α biosynthesis. Br. J. Pharmacol. **135**, 520–536
42 Seko, Y., Takahashi, N., Tobe, K., Kadowaki, T. and Yazaki, Y. (1997) Hypoxia and hypoxia/reoxygenation activate p65PAK, p38 mitogen-activated protein kinase (MAPK), and stress-activated protein kinase (SAPK) in cultured rat cardiac myocytes. Biochem. Biophys. Res. Commun. **239**, 840–844
43 Ozaki, M., Deshpande, S. S., Angkeow, P., Suzuki, S. and Irani, K. (2000) Rac1 regulates stress-induced, redox-dependent heat shock factor activation. J. Biol. Chem. **275**, 35377–35383

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