Dual regulation of heat-shock transcription factor (HSF) activation and DNA-binding activity by H₂O₂: role of thioredoxin

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The heat-shock (HS) response is a ubiquitous cellular response to stress, involving the transcriptional activation of HS genes. Reactive oxygen species (ROS) have been shown to regulate the activity of a number of transcription factors. We investigated the redox regulation of the stress response and report here that in the human pre-monocytic line U937 cells, H₂O₂ induced a concentration-dependent transactivation and DNA-binding activity of heat-shock factor-1 (HSF-1). DNA-binding activity was, however, lower with H₂O₂ than with HS. We thus hypothesized a dual regulation of HSF by oxidants. We found that oxidizing agents, such as H₂O₂ and diamide, as well as alkylating agents, such as iodoacetic acid, abolished, *in vitro*, the HSF-DNA-binding activity induced by HS *in vivo*. The effects of H₂O₂ *in vitro* were reversed by the sulphhydryl reducing agent dithio-

threitol and the endogenous reductor thioredoxin (TRX), while the effects of iodoacetic acid were irreversible. In addition, TRX also restored the DNA-binding activity of HSF oxidized *in vivo*, while it was found to be itself induced *in vivo* by both HS and H₂O₂. Thus, H₂O₂ exerts dual effects on the activation and the DNA-binding activity of HSF: on the one hand, H₂O₂ favours the nuclear translocation of HSF, while on the other, it alters HSF-DNA-binding activity, most likely by oxidizing critical cysteine residues within the DNA-binding domain. HSF thus belongs to the group of ROS-modulated transcription factors. We propose that the time required for TRX induction, which may restore the DNA-binding activity of oxidized HSF, provides an explanation for the delay in heat-shock protein synthesis upon exposure of cells to ROS.

INTRODUCTION

Eukaryotic cells continuously produce reactive oxygen species (ROS) from multiple sources, including mitochondrial electron-transport-chain components and a variety of soluble proteins and enzymic systems, such as NADPH oxidase during phagocytosis [1]. Major ROS include superoxide, hydrogen peroxide (H₂O₂) and hydroxyl radical, as well as nitric oxide. ROS have been described as essential second messengers in several metabolic pathways and also in the transduction-signal cascade leading to the activation of a number of transcription factors, including the nuclear transcription factor κ B (NF κ B) and the activator protein-1 (AP-1). Thus, ROS control the inducible expression of a number of genes involved in cell growth and differentiation, inflammation and immune responses and cellular stress [2–6]. Post-transcriptional regulation is also influenced by ROS. The translation of ferritin-specific mRNA, for example, depends on the redox state of the iron-response element, RNA-binding protein [7].

However, at high concentrations, ROS are cytotoxic for cells, leading to irreversible damage to DNA, proteins and lipids. Thus, cellular homeostasis requires the intracellular concentrations and toxic activities of ROS to be finely balanced with the co-ordinated induction of proteins with radical-scavenging properties and protective activities, such as superoxide dismutase, ferritin, haem oxygenase, thioredoxin (TRX) and heat-shock proteins (HSP). The induction of genes coding for these proteins is a crucial step in the cellular response to oxidative stress.

Expression of the highly conserved HSP (or stress proteins) has been observed as an adaptative response to oxidative stress in eukaryotes and prokaryotes. HSP represent a family of

proteins containing both constitutively expressed and stress-inducible members, usually classified according to their molecular mass, structure, functions and main inducers. In unstressed cells, HSP function as molecular chaperones, contributing to the folding and assembly of nascent polypeptides and to protein transport and degradation, as well as preventing stress-mediated accumulation of misfolded or damaged proteins. Thus, HSP protect cells and tissues under a number of conditions associated with oxidative damage, such as ischaemia-reperfusion [8] or tumour necrosis factor α cytotoxicity [9,10].

Transcriptional induction of heat-shock genes requires the activation of the heat-shock factor (HSF), which binds to the consensus heat-shock element (HSE) located in the promoter region of the heat-shock (HS) genes [11–13]. In unstressed cells, the HSF exists in a non-DNA-binding, cytoplasmic form. Activation of HSF into a DNA-binding form is accompanied by oligomerization and nuclear translocation. To date, two HSF have been identified in mammalian cells, HSF-1 and HSF-2 [14].

Previous studies have reported that while ROS induce *Drosophila* and human HSF-DNA-binding activity, HSP were not synthesized, or only weakly so [15,16]. Based on the observation that the oxidation of conserved redox-reactive cysteines in the DNA-binding domains of mammalian transcriptional regulators, such as NF κ B and AP-1, modulates their DNA-binding activity [17], we hypothesized that alterations in the cellular redox state might also regulate, either positively or negatively, activation and binding of HSF. Indeed, HSF contains three cysteine residues within its DNA-binding domain [18].

Here we report that H₂O₂ exerted a dual effect in the human premonocytic line U937: on the one hand, H₂O₂ induced HSF-DNA binding activity *in vivo*, while on the other, H₂O₂ and other

Abbreviations used: DTT, dithiothreitol; HS, heat shock; HSE, heat-shock element; HSF, heat-shock factors; aHSF, activated HSF; HSP, heat-shock proteins; ROS, reactive oxygen species; TRX, thioredoxin; NF κ B, nuclear transcription factor κ B; AP-1, activator protein-1; IAA, iodoacetic acid; NEM, N-ethylmaleimide; 2-ME, 2-mercaptoethanol.

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oxidants reversibly inhibited HSF-DNA-binding activity, *in vitro*. This activity was recovered, *in vitro*, in the presence of excess thiol. Accordingly, we examined the possibility that the cellular redox-active protein, TRX, is the physiological mechanism responsible for restoring HSF-DNA-binding activity and thus regulating HSF activity during oxidative stress. The NADPH-dependent TRX system has been described as a general disulphide reductase with specific functions as a hydrogen donor for ribonucleotide reductase and other substrate-reducing enzymes [19–21]. TRX contributes to the regulation of enzyme and receptor activity via redox control involving reversible thiol–disulphide exchange [20,22]. We found that TRX, whose expression is increased by both HS and H₂O₂, also functions as an endogenous regeneration system for HSF during oxidative stress.

EXPERIMENTAL

Materials

[γ -³²P]ATP (5000 Ci/mmol), Hyperfilm and the DNA 5′-end-labelling kit were obtained from Amersham (Buckinghamshire, U.K.). Poly(dI-dC) and deoxyribonuclease-free BSA were obtained from Pharmacia Biotech (Piscataway, NJ, U.S.A.). Diazinedicarboxylic acid bis-(*N,N*-dimethylamide) (diamine), dithiothreitol (DTT), iodoacetic acid (IAA), *N*-ethylmaleimide (NEM), 2-mercaptoethanol (2-ME) and protease inhibitors were purchased from Sigma Biochemicals (St. Louis, MO, U.S.A.). A reduced form of recombinant human TRX and goat antibody against human TRX were obtained from IMCO (Stockholm, Sweden). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.). Polyclonal antibodies against HSF-1 and HSF-2, prepared as described by Sarge et al. [23], were a gift from Dr. R. Morimoto (Northwestern University, Evanston, IL, U.S.A.).

Cell culture and treatment

The human pre-monocytic cell line U937 was maintained mycoplasma-free in stationary cultures in RPMI 1640 medium (Gibco, Paisley, Scotland, U.K.) supplemented with 10 % (v/v) fetal calf serum (Gibco) and 1 % (v/v) glutamine (Gibco), at 37 °C, in a humidified atmosphere (95 % air/5 % CO₂). For HS, U937 cells were incubated in a water-bath at 44 °C for 20 min and immediately prepared for nuclear extracts. U937 cells were treated for 1 h at 37 °C with concentrations of H₂O₂ ranging from 0.2 to 5 mM. In all other experiments U937 cells were exposed to 2 mM H₂O₂ for 1 h unless otherwise specified.

Preparation of nuclear and cytosolic extracts and gel mobility-shift assays

Preparation of nuclear-protein extracts was adapted from the method described by Dignam et al. [24]. Briefly, cells were collected, kept on ice for 10 min in a hypotonic lysis buffer containing 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and 1 µg/ml each of antipain, leupeptin and pepstatin. Nuclear pellets were resuspended in an extraction buffer [10 mM Hepes, pH 7.9/400 mM NaCl/1.5 mM MgCl₂/0.1 mM EDTA/0.5 mM DTT/0.2 mM PMSF/5 % (v/v) glycerol] with constant mixing for 30 min at 4 °C. The samples were centrifuged at 65000 *g* for 15 min at 4 °C and the supernatant dialysed against a buffer containing 20 mM Hepes (pH 7.9), 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 20 % (v/v) glycerol. Samples were quickly frozen in liquid nitrogen and stored at –80 °C. Nuclear extracts were then analysed for HSE-binding activity by gel mobility-shift assays. Binding reactions were performed for 30 min at 25 °C by adding

5 µg of nuclear proteins to a mixture containing 10⁶ c.p.m. of [γ -³²P]ATP end-labelled, double-stranded HSE oligonucleotide (5′GCCTCGAATGTTTCGCGAAGTT3′) in 15 µl of dialysis buffer containing 2 µg of poly(dI-C) and 10 µg of BSA. For the competition experiments, either a 100-fold molar excess of non-radioactive HSE or a 100-fold molar excess of the tumour-promoting agent responsive element oligonucleotide (5′CTTGIGAGTCATTCC3′) was used. Samples were electrophoresed on a non-denaturing 4 % polyacrylamide gel, dried and autoradiographed. In experiments aiming to identify which HSF was activated by HS or H₂O₂, nuclear proteins were pre-incubated for 15 min with a 1:50 (v/v) dilution of polyclonal rabbit antibodies against HSF-1 or HSF-2.

Oxidoreductive reactions *in vitro*

The nuclear extracts of heat-shocked cells, which therefore contain activated HSF, were also treated with either H₂O₂ (1 mM), diamine, IAA or NEM (10 mM each) for 15 min at room temperature. Reduction was obtained by adding for an additional 15 min various reducing reagents such as DTT (10 mM), 2-ME [1 % (v/v)] or TRX (80 µM).

Analysis of TRX expression

U937 cells were incubated for different times with H₂O₂ (2 mM). Cells were washed and lysed, and aliquots corresponding to equal amounts of proteins (70 µg) were resolved by SDS/PAGE, according to the method of Laemmli [25], using 15 % polyacrylamide, then transferred to nitrocellulose. Samples were probed with a goat polyclonal antibody against human TRX and bound antibodies were revealed with an anti-(goat IgG–alkaline phosphatase) conjugate (Sigma) in the presence of 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) as the substrate (Sigma).

Analysis of protein synthesis

In these experiments, RPMI 1640 was replaced by RPMI 1640 without methionine (Gibco). U937 cells were incubated in a water-bath at 44 °C for 20 min, followed by a recovery period of 2.5 h at 37 °C. Labelling was performed with [³⁵S]methionine (6 µCi/ml) (specific activity > 1000 Ci/mmol; Amersham) for 90 min at 37 °C. U937 cells were incubated with H₂O₂ (2 mM) for 4, 6, 8 and 16 h at 37 °C. [³⁵S]Methionine was added to the cells for the last 90 min of treatment. Aliquots corresponding to equal cell numbers were resolved by SDS/PAGE (with 10 % polyacrylamide) and revealed by autoradiography. Hsp70 expression was characterized by Western blotting. Proteins were electrophoresed, transferred to nitrocellulose membranes and probed with a mouse monoclonal antibody against the human inducible hsp70 (SPA810, StressGen Biotechnologies, Victoria, Canada). Bound antibodies were revealed with anti-(mouse IgG–peroxidase) conjugate (Sigma) in the presence of H₂O₂ and 4-chloro-1-naphthol (Sigma).

RESULTS

Inducible HSF-1-DNA-binding activity in heat-shocked and H₂O₂-treated cells

The activation of HSF and its DNA-binding capacity can be determined by gel mobility-shift assays using the synthetic oligonucleotide containing the consensus HSE-binding site from the human HSP promoter. Figure 1(A) shows HSE-binding activities detected in nuclear extracts prepared from U937 cells immediately after HS (20 min, 44 °C) or exposure to 2 mM H₂O₂

Figure 1 HSF-1 is activated in U937 cells exposed to H_2O_2

(A) U937 cells were exposed to HS (lanes 2–4) or to H_2O_2 (2 mM) for 1 h at 37 °C (lanes 5–7). Nuclear proteins (5 μ g) were subjected to gel mobility-shift assay using [32 P]HSE. The specificity of HSF-HSE-binding activity was controlled by competition experiments with a 100-fold molar excess of cold HSE (lanes 3 and 6) or tumour promoting agent responsive element (TRE) (lanes 4 and 7). Both HS and H_2O_2 induced HSF-HSE binding, but less with H_2O_2 than with HS. (B) Nuclear proteins prepared from cells exposed to HS (lanes 2–4) or to H_2O_2 (lanes 5–7) were pre-incubated with polyclonal antibodies specific for either HSF-1 (lanes 3 and 7) or HSF-2 (lanes 4 and 6). Both stresses activated HSF-1.

(1 h, 37 °C). Extracts from heat- and H_2O_2 -exposed cells both demonstrated HSE-binding activity, albeit of a different magnitude (compare lanes 2 and 5). Control-cell extracts contained no detectable HSE-binding activity (lane 1). The specificity of HSE-binding activities induced by HS or H_2O_2 was tested by competition experiments with a 100-fold molar excess of cold HSE or tumour-promoting agent responsive element. Only competition with HSE prevented the binding of HSF to [32 P]HSE (Figure 1A, lanes 3 and 4 for HS and lanes 6 and 7 for H_2O_2).

In order to establish which HSF (HSF-1 or HSF-2, two closely related members of the human HSF gene family) was involved in the H_2O_2 -inducible HSF-HSE-binding activity, nuclear extracts from HS- and H_2O_2 -treated cells were incubated with polyclonal antibodies specific for each HSF prior to gel mobility-shift assays (Figure 1B). Antibodies to HSF-1 interfered with both HS- and H_2O_2 -induced HSF-HSE-binding activity (lanes 3 and 7), whereas antibodies to HSF-2 had no effect (lanes 4 and 6).

Experiments performed with concentrations of H_2O_2 ranging from 0.2 mM to 5 mM indicated that the H_2O_2 -mediated HSF activation was concentration-dependent, with a threshold at

Figure 2 H_2O_2 induces dose-dependent activation and DNA binding of HSF

U937 cells were exposed to HS (lane 2) or to H_2O_2 at concentrations ranging from 0.2 to 5 mM (lanes 3–7). While H_2O_2 -induced HSF-HSE-binding activity was concentration dependent, the binding of HSF to HSE was still weaker than for HS even at the highest H_2O_2 concentration tested.

0.5 mM, but weaker with H_2O_2 than with HS, even at the highest concentration (Figure 2, compare lanes 2 and 7)

Reversible inactivation of HSF-HSE-binding activity by *in vitro* oxidation of nuclear extracts

As HSF-HSE-binding activity induced by H_2O_2 appeared qualitatively similar to, but quantitatively different from, that induced by HS, we hypothesized that H_2O_2 might, on one hand, favour the activation of HSF, and on the other, prevent its binding to HSE, by oxidatively altering HSF. We thus investigated the influence, on the DNA-binding capacity of HSF, of oxidative and others agents which can modify free sulphhydryl groups. Nuclear translocation of HSF was achieved by exposing the cells to HS. The resulting nuclear extracts were treated with alkylating agents, such as IAA or NEM, or oxidizing agents, such as H_2O_2 or diamine. The results are presented in Figure 3. Both alkylating agents (lanes 3 and 4) and oxidants (lanes 5 and 6) abolished HSF-binding to HSE.

TRX reverses the *in vitro* and *in vivo* effects of H_2O_2

The *in vitro* effects of H_2O_2 described above were completely reversed by further treatment with reducing agents such as 2-ME (Figure 3, lane 11) and DTT (lane 12), or by the endogenous reducing agent TRX (lane 13). Similar reversibility was observed with diamine (results not shown). In contrast, alkylation of nuclear extracts with IAA was not reversible by DTT or TRX (lanes 8 and 9).

We also tested the effects of TRX and DTT on HSF-HSE-binding induced by H_2O_2 *in vivo*. Nuclear extracts from cells exposed to H_2O_2 were treated with TRX or DTT, as mentioned above. Only TRX, but not DTT, was able to regenerate full HSF-binding activity in nuclear extracts from cells previously exposed to H_2O_2 (Figure 3, compare lanes 15 and 16 with lane 17). In the presence of TRX, the levels of HSF binding to HSE became similar to those observed with HS (compare lanes 17 and 14).

Figure 3 Oxidation or alkylation of cysteine residues abolishes HSF DNA-binding *in vitro*

Nuclear extracts prepared from heat-shocked cells were incubated at room temperature with IAA (10 mM), NEM (10 mM), diamine (10 mM) or H_2O_2 (1 mM) for 15 min, and then allowed to bind to [^{32}P]HSE for a further 30 min (lanes 3–6). To assess the reversibility of the inactivation of HSF, IAA- and H_2O_2 -treated nuclear extracts were subsequently incubated with DTT (10 mM) (lanes 8 and 12) or TRX (80 μM) (lanes 9 and 13), as well as, for H_2O_2 -treated extracts, with 2-ME [1% (v/v)] (lane 11) for a further 15 min before addition of the probe. In parallel, nuclear extracts from H_2O_2 -treated cells (lanes 15–17) were preincubated with 10 mM DTT (lane 16) or 80 μM TRX (lane 17). The reducing agents were able to restore oxidatively damaged HSF, but not alkylated HSF.

Figure 4 TRX expression is regulated by HS and H_2O_2

A Western blot for TRX is shown of U937 cells exposed to HS (4 h recovery) (lane 2) or to H_2O_2 , for the indicated time periods (lanes 3–7). Equal amounts of proteins were analysed. In addition to the constitutive expression of TRX in unstressed cells (lane 1), both stresses were able to up-regulate TRX expression.

TRX is induced by HS and by H_2O_2

Having found that TRX prevented the oxidant-induced inhibition of HSF, we hypothesized that TRX might represent a key factor in HSF regulation during oxidative injury *in vivo*. In order to be an efficient regulator under stressful conditions, a protein should be up-regulated during stress. U937 cells were exposed to HS or to H_2O_2 . Equal amounts of proteins were subjected to Western blot analysis using TRX-specific antibodies (Figure 4). Lane 1 shows the constitutive expression of TRX in U937 cells. TRX expression was induced by HS (lane 2) and by H_2O_2 in a time-dependent fashion (lanes 3–7).

Kinetics of H_2O_2 -mediated HSF-HSE-binding activity and HSP synthesis

According to the kinetics of induction of TRX, we hypothesized that the apparent lack of induction of HSP by oxidants, reported

Figure 5 Kinetics of H_2O_2 -mediated activation and DNA binding of HSF

U937 cells were exposed to HS (lane 2) or to H_2O_2 (2 mM) for the indicated time periods (lanes 3–9). Nuclear proteins (5 μg) were subjected to gel mobility-shift assay using [^{32}P]HSE. As expected, immediately after HS, HSF-HSE-binding activity was observed. In contrast, H_2O_2 -induced HSF-HSE complexes were detected only after 90 min.

by others [15,16], as well as by ourselves in U937 cells exposed to H_2O_2 [26], was in fact only delayed for the duration of time required for TRX induction. U937 cells were thus incubated in the presence of H_2O_2 (2 mM), and after the indicated times, nuclear extracts were prepared and tested for HSF-DNA-binding activity. As shown in Figure 5, compared with HS, which immediately generated HSE-HSF complexes (lane 2), the effect of H_2O_2 was delayed, the maximal HSF-DNA-binding activity

Figure 6 Time-course of induction of HSP synthesis following exposure to H_2O_2

(A) SDS/PAGE analysis of protein synthesis by U937 cells after HS (20 min at 44 °C) (lane 2) or treatment with H_2O_2 (2 mM) for the indicated time-periods (lanes 3–6). Lane 1: control (C); a, actin. (B) Western blot analysis for hsp70 under similar conditions to (A). HSP expression was detected 4 h after HS, and 6–8 h after exposure to H_2O_2 .

being observed at 2 h (lane 8). At this time-point, the H_2O_2 -induced HSF-DNA-binding activity was equally as efficient as it was for HS at 20 min (compare lanes 8 and 2).

We have previously reported that H_2O_2 did not induce HSP synthesis in U937 cells [26]. However, we had only investigated the early time-points after H_2O_2 exposure. Exposure of U937 cells for 20 min at 44 °C, followed by a 4 h recovery period at 37 °C, induced the classical HSP (hsp70, 90 and 110), whereas exposure to H_2O_2 (2 mM) for 4 h did not induce HSP synthesis (Figures 6A and 6B, compare lanes 2 and 3). Time-course experiments for protein synthesis indicated that there was indeed no induction of HSP up to 4 h after exposure to H_2O_2 , while the H_2O_2 -mediated induction of HSP synthesis (hsp70 and hsp90) started after 6 h incubation with H_2O_2 (lane 4), reached a peak at 8 h (lane 5) and disappeared after 16 h (lane 6). Hsp70 induction was confirmed by Western-blot analysis with specific monoclonal antibodies against hsp70 (Figure 6B).

DISCUSSION

The effects of oxidants on the HS response have been studied by a number of groups, including ours, often with conflicting results, suggesting that there is a multi-step and complex regulation of this response according to the cell type, the particular oxidant used, the subcellular location of its production and the time allowed for recovery from stress [16,26,27]. We thus investigated the effect of the membrane-permeant H_2O_2 on HSF

activation and binding and on HSP synthesis. Our results indicate a dual regulation of HSF by oxidants. This dual regulation appears to be under the control of TRX, a small ubiquitous protein containing two redox-active cysteine residues in the active site, whose reduced form functions as a hydrogen donor for the reduction of protein disulphides [27].

We first confirmed that H_2O_2 led to HSF activation in human cells, and specifically, to the activation of HSF-1, which is also the type of HSF activated by thermal stress, while HSF-2 regulates the activity of HS genes under non-stressful conditions, such as differentiation and development [28]. The activation of HSF by oxidants is selective for the type of oxidant, H_2O_2 being effective while superoxide is not [29]. The selectivity of oxidants in the activation of transcription factors may be a general phenomenon, since it has also been described for NF κ B, which, like HSF, is activated by H_2O_2 but not by superoxide [30].

This is not the only similarity in the regulation of HSF and other transcription factors by the redox state. Indeed, while inducing *in vivo* HSF activation, nuclear translocation and some degree of HSF-DNA binding, H_2O_2 oxidatively modified the resulting activated HSF (aHSF) *in vitro*, which prevented its binding to HSE. We propose that this inhibition of HSF-DNA-binding resulted from oxidation of cysteine residue(s) within the DNA-binding domain of HSF-1. NF κ B and AP-1 also harbour conserved cysteines within their DNA-binding sites, which have been proposed as the target for the modulatory effects of oxidants and reducing agents on those transcription factors [31–36]. The effects of oxidants on the DNA-binding activity of these transcription factors are modulated by intracellular thiols which, in turn, are regulated by glutathione and TRX. Interestingly, these antioxidants exert differential effects on NF κ B and on AP-1 [37–40].

The involvement of a redox mechanism in the HS signal-transduction pathway has recently been suggested by Huang et al. [41], who showed that the presence of DTT during stress inhibited the HS response. Thus, in order to test the involvement of sulphhydryl groups in HSF-HSE complex formation, we examined the effects, *in vitro*, of a number of redox- and thiol-reactive agents on HSF-DNA-binding activity. In particular, we investigated the role of TRX, a molecular chaperone which might catalyse, depending on the redox environment, the isomerization, oxidation or reduction of protein thiols/disulphides [19]. We found that oxidation-mediated inhibition of HSF-DNA-binding was reversed by exogenous reducing agents, such as DTT, 2-ME or TRX, while the alkylation-mediated inhibition was irreversible, suggesting that one or more reduced cysteine residue(s) within the DNA-binding domain of HSF are critical for binding. Interestingly, not all HSF contain cysteine residues within their DNA-binding region: for example, human HSF-2 or yeast HSF do not [42,43]. The selective activation of one or the other HSF might thus depend upon the molecular structure of HSF: human HSF-1 is regulated by redox modifications while HSF-2, in which cysteines are replaced by threonine or tyrosine residues, would be regulated by phosphorylation. The precise involvement of cysteine residues in HSF-HSE-binding activity should be confirmed by site-directed mutagenesis of these residues.

TRX is involved in the regeneration of cellular proteins inactivated by oxidative stress *in vivo* [44]. Accordingly, reduced human recombinant TRX prevented the inhibition of HSF-HSE-binding induced by oxidation *in vivo*, which was not the case for DTT. It is known that TRX, as a protein disulphide reductase, reacts about 10^5 times faster than DTT [45]. However TRX is itself an enzyme which is also regulated by oxidoreduction of SH groups [45], and in the absence of other reductants, TRX

Figure 7 A model for the redox regulation of HSF activation and DNA-binding activity by H_2O_2

See text for details.

may be inactivated by SH-group oxidation. While, *in vivo*, TRX is maintained in its reduced form by TRX reductase and NADPH [46], in our experiments, the amount of DTT contained in the commercial incubation mixture was probably enough to keep TRX reduced.

Both DTT and TRX prevented the H_2O_2 -induced DNA-binding activity of HSF when added before H_2O_2 (results not shown), suggesting that these reductants scavenge H_2O_2 . It has been proposed that TRX not only functions *in vivo* as a proton donor for numerous proteins, but also scavenges ROS (in particular H_2O_2 and hydroxyl radicals) and reactivates denatured proteins that contain mispaired disulphide bonds [47]. However, while the re-folding activity of TRX has been clearly demonstrated [48], this is not the case for its scavenging activity. TRX may regulate the scavenging activity of other antioxidants by facilitating proton transfer [49], or exert a direct effect as a free-radical quencher [47,50,51].

However, TRX expression was increased during HS and oxidative stress. The inducible expression of TRX in human cells by both HS and oxidative stress leads us to suggest that TRX might be a new member of the stress-protein families. Interestingly, the recently published sequence of the promoter region of human TRX reveals the presence of many regulatory elements compatible with HSF-responsive elements [52]. We propose that the time required for TRX induction provides an explanation for the lack of HSP synthesis upon exposure of cells to H_2O_2 , despite the activation of HSF, as reported in a number of studies [15,16]. Indeed, the time-dependent induction of TRX by H_2O_2 correlated well with the increase in HSF-DNA-binding activity observed after 120 min of exposure of cells to H_2O_2 . Previous studies were usually limited to 4 h of recovery after stress, a period which in U937 cells, for example, was insufficient to allow the H_2O_2 -mediated induction of HSP synthesis.

Figure 7 schematically summarizes the data presented here and their interpretation. HSF is maintained inactivated *in vivo* in the cytoplasm by association with a chaperone, which might be

hsp70 (inactivated HSF, iHSF) [53,54]. Upon exposure to oxidative stress, hsp70 dissociates from HSF favouring its trimerization and nuclear translocation (aHSF). However, cysteine residue(s) in the DNA-binding domain of HSF may also be a substrate for ROS leading to oxidation of HSF (aHSF-oxi), which is unable to bind HSE. Early after oxidative stress, only a small amount of aHSF is functional, while TRX is being induced ('early antioxidants', aiming at radical scavenging by direct interaction with ROS or regulation of other antioxidants and protection of HSF and other transcription factors). After a prolonged recovery time, up-regulation of TRX catalyses the transformation of aHSF-oxi into the reduced form (aHSF-red), which efficiently interacts with HSE. Then, transcriptional activation of HS genes occurs until sufficient amounts of HSP are synthesized ('late protection-repair', i.e., proteins involved in overall protein repair, such as HSP).

In conclusion, the HS response, which depends on the optimal transcriptional activity of HSF, appears to be finely tuned by a balance between oxidants and antioxidants. This equilibrium depends on the type of stress, its consequences for the cellular redox state and the time allowed for recovery. HSF belongs to the ROS-modulated transcription factors and is regulated at two distinct levels: induction/activation and DNA binding. In addition to phosphorylation, which is essential for the transcriptional activity of some HSFs [55], redox modifications represent another efficient mechanism for controlling the HS response.

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