Tobacco-smoke-inducible human haem oxygenase-1 gene expression: role of distinct transcription factors and reactive oxygen intermediates

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Exposure of eukaryotic cells to a variety of reactive-oxygen-intermediate (ROI)-mediated sources of cellular injury, including heavy metals and UV radiation, induces the expression of heat-shock (HS) and stress-related genes among which is a 32–34 kDa protein identified as inducible haem oxygenase-1 (HO-1). We previously showed that tobacco smoke (TS), a potent source of oxidants leading to oxidative stress, induces both HS proteins (HSPs) and HO-1 in normal human monocytes. Here we investigated the induction mechanisms of human HO-1 gene expression by TS in the human premonocytic line U937. Northern blotting and flow cytometry revealed a dose- and time-dependent induction of HO-1 mRNA and protein by TS. In order to clarify the role of transacting factors in this induction, electrophoretic mobility-shift analysis was performed with nuclear extracts from control, TS-, cadmium (Cd)- or H₂O₂-exposed cells, incubated

with consensus elements and binding sites of the promoter region of HO-1 [heat-shock factor (HSF), nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1)] and the cadmium-responsive element (CdRE) isolated by Takeda, Ishizawa, Sato, Yoshida and Shibahara [(1994) *J. Biol. Chem.* **269**, 22858–22867]. We report an inhibition of NF- κ B activation by TS, no effect on AP-1 and a strong activation of CdRE-binding activity, whereas cadmium chelation from TS only partially prevented HO-1 induction. H₂O₂ also activated the CdRE-binding activity, and pretreatment with *N*-acetyl-L-cysteine, which replenishes the intracellular levels of GSH, suppressed, in TS-treated cells, both the CdRE-binding activity and the increased HO-1 expression.

Key words: binding activity, cadmium-responsive element, oxidative stress.

INTRODUCTION

Expression of stress-related genes are particularly induced following exposure to thiol-reactive compounds [1], reactive oxygen intermediates (ROI) [2–4], heavy-metal cations [5–7], UV radiation [8,9] and other chemical initiators of cellular stress responses, such as H₂O₂ or lipopolysaccharides [10,11]. These stimuli all induce the expression of a 32–34 kDa protein identified as the microsomal inducible haem oxygenase-1 (HO-1), whose regulation is related to heat-shock proteins (HSPs) in rodents, but not in humans ([12–15]; a review [16]), showed that bile pigments produced by haem degradation catalysed by HO-1 can function as antioxidants, while Keyse and Tyrrell [2] demonstrated that HO-1 is involved in the general cellular defence mechanisms against oxidative injury.

Tobacco smoke (TS) contains numerous toxic substances, including heavy metals, and is also a potent source of oxidants [17,18]. TS, in aqueous solutions, induces lipid peroxidation [19,20], DNA-single-strand breaks [21,22] and decreases the levels of GSH and protein thiols [20]. Although the precise mechanisms by which cells sense and respond to a specific environmental stress to activate the transcription of appropriate target genes are still not completely understood, stimulation of HO-1 expression is controlled primarily at the level of gene transcription. More generally, the activation of gene transcription plays a vital role in the ability of all organisms to induce a

protective response to environmental stresses. Upon exposure to a specific stress, cells activate the transcription of genes encoding proteins that either offer protection from stress, or repair stress-induced intracellular damage. In the case of HO-1, a growing number of transcription factors and their cognate binding sites that mediate transcriptional activation of the gene in response to distinct agents has been described. Alam et al. [5,23] demonstrated that activation of the mouse HO-1 gene by phorbol esters, haem and cadmium (Cd) is mediated by a fragment, located 4 kb upstream of the transcription initiation site and composed of two elements that interact with the Fos/Jun [activator protein-1 (AP-1)] family of transcription factors, while a *cis*-acting element responsible for Cd-mediated induction of the human HO-1 gene has been identified by Takeda et al. [6]. In contrast, haem induction of human HO-1 gene has been shown to be mediated by nuclear factor κ B (NF- κ B) and AP2 transcription factor activation [24].

In the present study we investigated the mechanisms through which TS-induced HO-1 expression is modulated, and the different transcription factors involved in response to TS exposure. In order to address this issue, we incubated nuclear extracts from control and TS-treated U937 cells with radio-labelled synthetic oligonucleotides corresponding to the following stress and metal responsive elements: HSE, NF- κ B-RE, AP-1-RE, and CdRE, in the promoter region of the human HO-1 gene [25] and examined the formation of sequence-specific DNA–

Abbreviations used: ROI, reactive oxygen intermediate(s); HS, heat-shock; HSP, heat-shock protein; HO-1, haem oxygenase-1; TS, tobacco smoke; HSF, heat-shock factor; HSE, HS element; RE, responsive element for NF- κ B (nuclear factor κ B) and AP-1 (activator protein-1); CdRE, cadmium (Cd)-responsive element; NAC, *N*-acetyl-L-cysteine; FCS, fetal-calf serum; MBB, monobromobimane; $\Delta\psi_m$, mitochondrial membrane potential; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; EMSA, electrophoretic gel mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cba, constitutive HSE binding activity; iba, inducible forms of DNA binding activity; κ B, inhibitory κ B.

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protein complexes. Our results suggest that CdRE functions as a general stress-response element in human cells and, in particular, might mediate the regulation of HO-1 gene expression by redox modulation.

EXPERIMENTAL

Reagents

[γ - 32 P]ATP (5000 Ci/mmol), Hyperfilm and the DNA 5' end labelling kit were obtained from Amersham. Poly(dI-dC) and DNase-free BSA were obtained from Pharmacia Biotech. Protease inhibitors, CdCl₂ (1, 10, 50 and 100 μ M), H₂O₂ (2 mM), *N*-acetyl-L-cysteine (NAC; 20 mM) and Chelex-100 (1 g in 4 ml of aq. TS extracts) were purchased from Sigma.

Cells and media

The human premonocytic line U937 was maintained in stationary cultures in RPMI 1640 medium (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.), supplemented with 10% (v/v) fetal-calf serum (FCS), 2 mM glutamine, 25 mM Hepes, 25 μ g/ml ampicillin and 120 μ g/ml penicillin (all from Gibco BRL) in a humidified atmosphere (air/CO₂, 19:1).

Exposure to TS

A peristaltic pump-smoke machine (Heinrich Borgwaldt RM1/G, Hamburg, Germany) was used to generate TS-bubbled PBS, from mainstream smoke of standard research cigarettes (reference 2R1; University of Kentucky, Lexington, KY, U.S.A.) through a puffing mechanism related to the human smoking pattern (1 puff/min; 1 puff = 35 ml; each puff of 2 s duration). In our experiments, the aqueous smoke fraction of one cigarette corresponded to 10 puffs (350 ml) bubbled into 5 ml of PBS. The final dilutions are expressed as puffs/ml of culture medium. Dose-response experiments were realized between 0.03 and 0.48 puff/ml. U937 cells were exposed to TS-bubbled PBS overnight, then recovered by pipetting and washed twice in PBS before nuclear extract preparation. In selected experiments, U937 cells were exposed to CdCl₂ or H₂O₂, or pre-exposed to Chelex-100 or NAC as indicated.

Isolation of RNA and Northern-blot analysis

Total RNA was prepared from U937 cell line by using a single-step procedure [26]. Samples of total RNA (10 μ g) were fractionated in 0.8%-agarose/formaldehyde denaturing gels, then transferred to a nylon membrane (Hybond N+; Amersham) and hybridized at 65 °C under standard conditions with 10⁸ c.p.m./ml of random-primed (Boehringer-Mannheim) [α - 32 P]dCTP-labelled probe. The cDNA probe was generated from a mixture of TS-treated U937 total RNA in 25 μ l of reverse-transcriptase reaction mixtures using sequence-specific oligonucleotide primers: 5'-CCGCAACCCGACAGCATGCC-3' (nucleotide position 90) and 5'-CCGCTTCACATAGCGCTGC-3' (nucleotide position 414) from the 5'-translated region of HO-1 cDNA [27]. Human HO-1 mRNA was detected at 1.9 kb.

Detection of HO expression by flow cytometry

U937 cells were exposed to 0.06 to 0.48 puff/ml of TS for 16 h. Permeabilized cells were incubated with anti-(human HO-1) monoclonal antibody OSA-110E from StressGen Biotechnologies Corp., Victoria, BC, Canada, at a dilution of 1:100 in PBS supplemented with 1% BSA (PBS/BSA) and stained with rabbit

anti-mouse IgG conjugated to FITC (1:30 dilution; Dako, Glostrup, Denmark) as previously described in detail [28]. Analysis was performed using an Epics Elite flow cytometer (Coulter, Miami, FL, U.S.A.) equipped with a single 488 nm argon laser. In all cases, a total of 5000 cells per sample were analysed in list mode through a 525 nm filter (Elite software version 4.02).

Flow-cytometric analysis of GSH content and mitochondrial membrane potential

The non-fluorescent thiol probe monobromobimane (MBBr) obtained by Molecular probes was used as described in [29]. This compound binds avidly to thiol groups to form blue fluorescent conjugates. MBBR was made up as a 4 mM stock solution in 100% ethanol. Under the staining conditions used, fluorescence is highly correlated with the cellular content of GSH [30]. In parallel with GSH content, we also analysed, by confocal microscopy, the mitochondrial membrane potential ($\Delta\psi_m$) of untreated and TS-treated U937 cells using 5,5',6,6'-tetrachloro-1,1',3,3',-tetraethylbenzimidazolcarbocyanine iodide (JC-1) probe as described in [31,32]. In these experiments, mitochondrial membrane depolarization is associated with a large shift in JC-1 fluorescence emission from red to green.

Preparation of nuclear extracts and electrophoretic gel mobility-shift assays (EMSA)

Preparation of nuclear extracts was adapted from the method of Dignam et al. [33]. Briefly, cells were collected and kept on ice for 10 min in hypotonic lysis buffer containing 10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF and 1 mg/ml each antipain, leupeptin and pepstatin. The cells were homogenized in a Dounce homogenizer and the nuclei pelleted at 3000 rev./min (800 g) and resuspended in extraction buffer [10 mM Hepes, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF and 5% (w/v) glycerol] with constant agitation at 4 °C for 30 min. The samples were centrifuged at 14000 rev./min (17500 g) for 15 min at 4 °C, and the supernatant was dialysed against buffer containing 20 mM Hepes, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF and 20% glycerol. Samples were quick-frozen in liquid nitrogen and stored at -80 °C. Protein concentration was determined with the bicinchoninic acid ('BCA') protein assay kit (Pierce, Rockford, IL, U.S.A.). Nuclear extracts were then analysed for oligonucleotide element binding activity by EMSA. Binding reactions were performed for 30 min at 25 °C by adding 5 μ g of nuclear proteins to a mixture containing 106 c.p.m. of [γ - 32 P]ATP end-labelled, double-stranded oligonucleotide in 15 μ l of dialysis buffer containing 2 μ g of poly(dI-dC) and 10 μ g of BSA: HSE (5'-GCCTCGAATGTTTCGCGAAGTT-3'), NF- κ B (5'-AGTTGAGGGGACTTTCCCAGGC-3'), AP-1 (5'-GCTGAGTCA-CCAGTGCC-3') and CdRE (5'-AGGCGGATTTTGCTAG-ATTT-3'). For the competition experiments, a 100-fold molar excess of non-radioactive oligonucleotide of interest was added. Samples were electrophoresed on non-denaturing 4%-(w/v)-polyacrylamide gels, dried, then autoradiographed.

RESULTS

Effects of TS, Cd and H₂O₂ on HO-1 mRNA accumulation

The level of mRNA was measured by Northern-blot analysis to confirm that TS and Cd exposure affects gene expression. A 32 P-labelled oligonucleotide probe specific for HO-1 mRNA was

Figure 1 Northern-blot analysis of HO-1 mRNA in U937 cells exposed to TS, Cd and H₂O₂

Upper panel: each lane contained 10 μ g of total RNA prepared from U937 cells incubated with 0.24 puff/ml for 2 h and 4 h (lanes 1 and 2), with 100 μ M CdCl₂ for 2 h and 4 h (lanes 3 and 4), with 2 mM H₂O₂ for 2 h and 4 h (lanes 5 and 6) or untreated (lane 7). The hybridization probe was the ³²P-labelled PCR fragment (nucleotide residues 90–414) derived from human HO-1 cDNA [27]. Lower panel: the membrane was re-probed with a ³²P-labelled DNA fragment homologous with GAPDH mRNA.

Figure 2 Effects of TS and CdCl₂ on HO-1 expression in U937 cells

(A) U937 cells were exposed to increasing concentrations of TS (0.03–0.48 puff/ml, 16 h), then permeabilized and labelled with anti-(human HO-1) antibody. Analysis of immunofluorescence was performed by flow cytometry. Expression of HO-1 increased after exposure to moderate concentrations of TS [0.06 puff/ml (broken curve) and 0.12 puff/ml (continuous curve)], but decreased at high concentrations (0.48 puff/ml; **bold** curve). Hatched curve, control cells. (B) U937 cells were exposed to Cd (10 μ M, 16 h) then labelled with anti-human HO-1. **Bold** curve, 10 μ M Cd for 16 h; stippled curve, control cells. ($n = 3$)

hybridized to a membrane that contained RNA prepared from the control U937 cell line and U937 cells exposed to 0.24 puff/ml of TS, 100 μ M of CdCl₂ or 2 mM of H₂O₂ for 2 h and 4 h (Figure 1). We noted that the TS-induced HO-1 mRNA expression is time-dependent: 4 h of incubation give greater mRNA accumulation (Figure 1, lanes 1 and 2). For CdCl₂, the induced HO-1 mRNA expression is practically the same at 2 h as at 4 h (Figure 1, lanes 3 and 4). The conditions of time and dose of H₂O₂ failed

to express any HO-1-induced mRNA (Figure 1, lanes 5 and 6). Results for untreated cells are shown in Figure 1, lane 7.

The intensity of the 1270 bp signal detected by a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as a standard for HO-1 mRNA quantification, in order to take into account variations in the initial amounts of mRNA present in each sample.

Effects of TS and Cd on HO-1 protein expression

U937 cells were exposed to different concentrations of TS (0.06–0.48 puff/ml) or to Cd (1–10 μ M) during 16 h, and HO-1 expression was analysed by flow cytometry. Exposure of U937 cells to TS resulted in an increased expression of HO-1 protein as compared with control cells, confirming previous reports from our [34] and other laboratories [18] using other techniques. HO-1 expression was maximal with 0.06 puff/ml, while at higher concentrations of TS, HO-1 induction was inhibited, completely stopping at 0.48 puff/ml (Figure 2A). Figure 2(B) shows that HO-1 was also induced by Cd (10 μ M) for 16 h.

This TS-dependent dual increase and decrease was also observed at shorter incubation times with higher concentrations of TS; we tested several doses of TS varying from 1 to 6 h (0.48 puff/ml for 1 h, 0.24 puff/ml for 2 h and 0.12 puff/ml for 6 h). The time point for which HO-1 begins to be induced is 0.24 puff/ml for 2 h and the maximum is reached at 0.12 puff/ml for 6 h. The dose of 100 μ M CdCl₂ for 2 h showed the same curve of HO-1 induction as that obtained at 10 μ M for 16 h.

Effects of TS on U937-cell GSH content and $\Delta\psi_m$

As shown in Figure 3, both parameters were altered as early as 2 h after exposure to TS as revealed by confocal image. Depletion of GSH by TS treatment is symbolized by changes in the fluorescence: the cells are coloured in blue after TS treatment (Figure 3A versus 3B). JC-1 emitted red fluorescence in control cells and green fluorescence in cells with depolarized membrane mitochondria after TS exposure (Figure 3C versus 3D).

The effects of TS on GSH content have been previously described in different cell types [35].

Effects of TS on HSF, NF- κ B, AP-1 binding factors

Among the multiple regulatory elements of HO-1, we first explored HSF, NF- κ B and AP-1 [24]. In order to identify the *cis*-acting elements responsible for TS-mediated induction of the HO-1 gene, we first performed EMSA using a synthetic oligonucleotide containing the radiolabelled consensus HSE-binding site incubated with nuclear extracts from TS-exposed cells (0.24 puff/ml, 2 h). We observed typical HSF–HSE complexes (Figure 4A, lane 2). Under our experimental conditions, both the constitutive HSE binding activity (cba) and the inducible forms of DNA binding activity (iba) were suppressed by an excess of unlabelled HSE (Figure 4A, lane 3).

Binding of NF- κ B was next examined using a synthetic oligonucleotide containing the consensus NF κ B binding site in TS-exposed cells. We observed an inhibition of the complexes and a loss of the upper band in TS-exposed cells (Figure 4B, lane 2) as compared with control cells (Figure 4B, lane 1). Inhibition of NF- κ B activation along with HSF activation has been reported [36] and is not specific of TS. Other stresses, including HS, arsenite and cyclopentanone prostaglandins, also inhibit NF- κ B [37]. Specificity of NF- κ B binding was tested by competition

Figure 3 Effects of TS on U937-cell GSH content and $\Delta\psi_m$

Cells were double-stained with MBBR and JC-1. Confocal appearance of U937 cells before and after TS revealed changes of colour reflecting (1) a depletion in GSH content (**A** to **B**) and (2) a decrease in $\Delta\psi_m$ (**C** to **D**). The scale bars represent 10 μm .

experiments with a molar excess of 100-fold non-radioactive NF- κ B (Figure 4B, lane 3).

We also analysed nuclear protein extracts from TS-exposed and control cells with a specific oligonucleotide probe containing the binding site for AP-1. No modification of the pattern was observed under the same conditions, as described above (Figure 4C, lanes 1 and 2). These results strengthen those published by Takeda et al. [6], which indicate that AP-1 binding site is not directly involved in Cd-mediated activation of the human HO-1 gene. They showed that relative luciferase activities were induced by Cd in the THP-1 cell line transfected with plasmid containing CdRE. In contrast, no Cd-mediated increases were detected in the cells transfected with the plasmid containing AP-1 binding site but lacking CdRE fragment. It is still possible that treatment of cells exposed to stressors with longer or shorter TS incubation times should raise AP-1 levels, as has been reported in early genes [38] where increased binding of *fos* and *jun* to AP-1 oligonucleotide was observed only after 30 min of treatment with active oxygen.

Effects of TS on CdRE-binding activity

We next examined the possibility that TS-induced HO-1 expression was mediated by a Cd-responsive element (CdRE) localized about 4 kb upstream from the transcription initiation

site of the human HO-1 gene specifically responsible for Cd-mediated induction [6]. We examined whether nuclear factors specifically bind to this CdRE after exposure to TS. Cells were exposed to TS (0.24 puff/ml during 2 h), nuclear proteins extracted, the 20 bp oligonucleotide containing the radiolabelled core CdRE [6] added, and the binding activity was determined. The data are presented in Figure 5; increasing concentrations of nuclear proteins induced a dose-dependent binding complex (see lanes 2, 3 and 4 as compared with the control, lane 1); competition experiments confirmed this specificity (Figure 5, lane 5).

CdRE-binding activity is induced by Cd

Our next aim was to confirm the Cd-dependency of CdRE binding activity. Takeda et al. [6] have reported in EMSA experiments the presence of two constitutive retarded bands and have shown that CdRE binding activity was unchanged by Cd treatment under their conditions. However, in our experiments, we observed a specific CdRE iba when cells were treated with 100 μM Cd for 2 h (Figure 6, lane 3) as compared with 50 μM (lane 2) and non-treated cells (lane 1). This high concentration of Cd did not, however, affect cell viability as estimated by the percentage of lactate dehydrogenase release (results not shown); in other reported experiments [39] where cells were exposed to Cd,

Figure 4 Effects of TS on HSF, NF-κB and AP-1 binding activity

(A) U937 cells were exposed to TS (0.24 puff/ml, 2 h) and nuclear proteins (5 µg) subjected to EMSA in the presence of labelled HSE. A major inducible HSF-binding activity (iba) was revealed (lane 2), as compared with control cells (lane 1). Specificity was controlled by competition experiments with a 100-fold molar excess of unlabelled HSE (lane 3). Constitutive binding activity (cba) was present in control and in TS-treated cells. (B) Similar experiments as described for (A) were performed with a labelled sequence binding site for NF-κB. Basal NF-κB-binding activity (three specific bands) was present in the control (lane 1); by contrast, TS exposure inhibited NF-κB-binding activity, with the highest effect observed for the upper band (lane 2). The specificity of NF-κB-binding activity was controlled by competition experiments, with a 100-fold molar excess of non-radioactively labelled consensus sequence binding site for NF-κB (lane 3). (C) Similar experiments were also conducted with a binding site for AP-1 (20 bp element synthesized from HO-1 promoter). No specific iba was found, the binding activity being unchanged by exposure to TS (lane 1 compared with lane 2).

Figure 5 Effects of TS on CdRE-binding activity

U937 cells were exposed to TS (0.24 puffs/ml, 2 h). Increasing amount of nuclear (Nucl.) proteins were subjected to EMSA using the labelled synthetic CdRE as described. Iba was increased (lanes 2, 3 and 4, upper bands) when the cells were treated with TS as compared with controls (lane 1). Specificity was confirmed by competition experiments with a 100-fold molar excess of non-radioactively labelled CdRE (lane 5). The lower bands correspond to non-specific (ns) binding activity.

the medium was also supplemented with 100 µM CdCl₂ for much longer periods of time (8 or 24 h).

Different incubation times and different doses of inducers may differ other elements of transcription and signalling cascades as recently described for rat brain tumour cells [40].

Differential modulation of HO-1 expression by Cd and TS

Flow cytometry indicated that Cd-mediated HO-1 protein expression was abolished when using the Cd-chelating resin Chelex-

Figure 6 Effects of CdCl₂ on CdRE-binding activity

U937 cells were exposed to increasing doses of CdCl₂ (50–100 µM, 2 h). Nuclear proteins (5 µg) were subjected to EMSA using the labelled CdRE. A major iba was revealed with 100 µM (lane 3). No iba was found when using 50 µM (lane 2).

100 (Figure 7A), while depletion of Cd from TS by the same chelating agent did not affect HO-1 induction (Figure 7B). This suggests that Cd contained in TS was not the only agent responsible for HO-1 induction.

Effects of H₂O₂ on CdRE binding activity

As it has previously been shown that ROI are mainly responsible for TS-dependent DNA damage [18,21,41], we postulated that ROI, and in particular H₂O₂, might also represent causative agents in TS-dependent CdRE-binding activity. We thus examined the binding specificity to CdRE of nuclear extracts of cells treated with H₂O₂ (2 mM, 2 h). Incubation of nuclear extracts from H₂O₂-treated cells with the radiolabelled CdRE resulted in a specific iba (Figure 8A, lane 2) as compared with control cells (Figure 8A, lane 1), further supporting the hypothesis that CdRE-binding activity might be redox-dependent. Activation of binding activity does not always result in activation of corresponding transcription genes, as is shown in Figure 1; we did not observe mRNA accumulation after H₂O₂ treatment (Figure 1), although we observed an activation of CdRE binding activity. This phenomenon has been reported in NIH-3T3 cells [42], where oxidative injury activates the HSF but does not increase levels of HSPs.

Effects of NAC on TS-induced CdRE binding activity and HO-1 expression

NAC, a known antioxidant and a free-radical scavenger [43] increases intracellular GSH stores, which is of particular importance in the case of GSH depletion caused by TS exposure [35]. NAC, like GSH, is able to detoxify reactive electrophiles and free radicals either through conjugation or reduction reactions, and reduces ROI to less reactive products. Pre-incubation of nuclear extracts with 20 mM NAC before exposure to TS resulted in the loss of CdRE-binding activity (Figure 8B, lane 3) as compared with nuclear extracts from cells exposed to TS but not pre-incubated with NAC (Figure 8B, lane 2). Analysis of HO-1 expression by flow cytometry confirmed the inhibition

Figure 7 Effects of chelex on TS-induced HO-1 expression

(A) When cells were preincubated with Chelex-100 before being exposed to Cd ($10 \mu\text{M}$), inhibition of HO-1 induction was practically complete. (B) When cells were similarly preincubated with Chelex-100, but before being exposed to TS, there was no such inhibition.

Figure 8 Effects of H_2O_2 and NAC on CdRE-binding activity

(A) U937 cells were exposed to H_2O_2 (2 mM, 2 h). Nuclear proteins ($5 \mu\text{g}$) were subjected to EMSA using the labelled CdRE; H_2O_2 induced CdRE-binding activity (lane 2) as compared with control cells (lane 1). (B) When cells were pre-incubated with NAC (20 mM, 2 h), then exposed to TS (0.24 puff/ml, 2 h), the iba was inhibited (lane 3 compared with lane 2).

of HO-1 induction when cells were pre-incubated with NAC before exposure to TS (Figure 9).

DISCUSSION

In the present study we found that TS-induced HO-1 expression is modulated by the cellular redox state in the human premonocytic line U937 and suggest that the 20 bp human CdRE sequence plays a role in the regulation of HO-1 expression by ROI, notably those contained in TS. We also report the involvement of different *cis*-acting elements on the HO-1 promoter and their respective TS-iba. Accumulated evidence indicates a role for HO protein in cellular and tissue damage and suggests that HO-1 induction is a protective response against oxidative stress [44]. Several studies on the regulation of HO-1 gene expression have focused on the induction mediated by thermal stress [45], metal ions [6] or haem [46]. These studies revealed various *cis*-acting elements in the HO-1 promoter and indicated that HO-1 might be induced by distinct mechanisms depending on the induction stimulus.

We first analysed HSE binding activity and found a relevant TS induction of HSF activity. Okinaga et al. [45], who studied

Figure 9 Effects of NAC on TS-induced HO-1 expression

U937 cells were pre-incubated with NAC (2 mM, 1 h) then exposed to TS (0.24 puff/ml, 2 h). TS-induced HO-1 expression was also inhibited by NAC.

the induction of HO-1 by HS, demonstrated that the HSE sequence on human HO-1 promoter is potentially functional *in vitro*, though its function is repressed *in vivo* because of the presence of a purine-rich sequence on HO-1 promoter downstream from the HSE, which confers on it a silencing effect. We thus deduced that the observed TS-inducible HO-1 expression is likely to be mediated by pathways distinct from those of HSF.

NF- κ B normally exists in an inactive cytosolic complex specifically bound to inhibitory protein κ B (inhibitory κ B). Stimulation triggers the release of NF- κ B from κ B, resulting in NF- κ B translocation to the nucleus, where it activates the transcription of a number of genes encoding stress-response factors. We confirmed that TS inhibited the activation of NF- κ B in U937 cells [36] and thus excluded a possible mediation of the observed TS inducibility of HO-1 by NF- κ B activation.

Functional AP-1 binding sites within the HO-1 promoter have been identified, suggesting the involvement of the Jun/Fos family of transcription factors in mediating the induction of HO-1 gene transcription by multiple agents [23,47,48]. Our results, however, revealed that TS-mediated HO-1 induction occurred in absence of AP-1 activation.

In contrast we found that CdRE, isolated as a specific Cd-responsive element on the HO-1 promoter [6], responded both to TS and to ROI (H_2O_2), and that this CdRE-binding activity was inhibited by addition of the GSH-replenishing antioxidant agent NAC. The induction of CdRE-binding activity and HO-1 expression in response to TS was, however, stronger than with H_2O_2 or Cd alone. TS certainly contains a large variety of compounds which might combine synergistically to provoke a stronger induction of HO-1 expression than each compound taken alone (Figures 1,5 and 6). However, our results suggest that, among those compounds, multiple ROI derived from TS are the most likely to be responsible for GSH depletion and HO-1 expression in TS-exposed cells. This hypothesis is in good agreement with previous work from our laboratory on the role of ROI in TS with respect to the induction of either HSP expression or apoptosis. The depletion of intracellular thiols such as GSH (Figure 3) resulting from TS exposure and protein-thiol-group alterations, as also suggested by Kato et al. [49], might favour the activation of a redox-sensitive component of the CdRE-binding protein (CdRE-BP) pathway. Interestingly, also, the effects of other inducers of HO-1, such as haemin, cobalt-protoporphyrin and arsenite, whose effects are not mediated by ROI, do not activate CdRE-binding activity [6].

The effects of Cd itself on CdRE-binding activity might be, at least in part, mediated by ROI, as it is now generally recognized that ROI are the essential mediators of Cd toxicity [50]. Since Cd has a high affinity for sulphur, some toxic effects of Cd are results of reactions with essential thiol groups in proteins [51]. Cd shows chemical similarities to zinc, and it may affect the properties of zinc-containing nuclear transcription factors and enzymes such as Cu/Zn-superoxide dismutase, which is inhibited by the substitution of zinc with Cd [52,53], thus leading to an increase in ROI production and toxicity. Cd may, however, also mimic or modify a physiological mediator that would be involved in a signal-transduction system leading to phosphorylation or dephosphorylation of the putative CdRE-BP. This putative CdRE-BP may relate to the antioxidant responsive element (ARE)-BP [54]. ARE has been found, for example, in the promoter region of the mouse glutathione S-transferase, an ROI-detoxifying enzyme, and mediates the transcriptional activation of the gene by transacting factors binding to ARE [55]. A model of the ARE as a composite regulatory site, where multiple transcription factors interact was presented by the same authors [55], in which the ARE 'core' sequence RTGACNNGC (where R = G or A,

and N = G or A or T or C) is necessary to mediate an antioxidant response. Interestingly, CdRE also contains an ARE-like core sequence, and it is possible that the as-yet-unidentified CdRE-BP corresponds to the ARE-BP1 defined by Wasserman and Fahl [54,55].

We are particularly grateful to Dr M. Vayssier-Taussat for her help in cell culturing and in the preparation of nuclear extracts, to Dr M. Bachelet for lactate dehydrogenase determinations and critical review of the manuscript before its submission, and to Dr E. Mariéthoz for assistance in the artwork. We thank Professor Andrea Cossarizza for his contribution to mitochondrial analysis. We also thank Professor Rex Tyrrell, Department of Pharmacy and Pharmacology, University of Bath, Bath, U.K., for stimulating discussions. F.F. is supported by the Centre National de la Recherche Scientifique (CNRS), and B.S.P. is supported by Institut National de la Santé et de la Recherche Médicale (INSERM).

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Received 19 October 2000; accepted 14 November 2000