# Differential activation of heat-shock and oxidation-specific stress genes in chemically induced oxidative stress

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Post-ischaemic reperfusion increases the levels of the major heat-shock (stress) protein hsp 70 and of its mRNA by transcriptional mechanisms, and activates the binding of the heat-shock factor HSF to the consensus sequence HSE. In common with $CoCl_2$ treatment, post-ischaemic reperfusion increases the level of haem oxygenase mRNA, an indicator of oxidative stress, but $CoCl_2$ does not seem to induce the expression of the hsp 70 gene [Tacchini, Schiaffonati, Pappalardo, Gatti and Bernelli-Zazzera (1993) Lab. Invest. **68**, 465–471]. Starting from these observations, we have now studied the expression of two genes of the hsp 70 family and of other possibly related genes under conditions of oxidative stress. Three different chemicals, which cause oxidative stress by various mechanisms and induce haem oxygenase, enhance the expression of the cognate hsc 73 gene, but do not activate the inducible hsp 70 gene. Expression of the other genes that have been studied seems to vary in intensity and/or time course, in relation to the particular mechanism of action of any single agent. The pattern of induction of the early-immediate response genes c-fos and c-jun observed during oxidative stress differs from that found in post-ischaemic reperfused livers. Oxidative-stress-inducing agents do not promote the binding of HSF to its consensus sequence HSE, such as occurs in heat-shock and post-ischaemic reperfusion, and fail to activate AP-1 (activator protein 1). With the possible exception of Phorone, the oxidative stress chemically induced in rat liver activates NFkB (nuclear factor kB) and AP-2 (activator protein 2) transcription factors.

## INTRODUCTION

Blood reperfusion of the liver after ischaemia *in situ* (warm ischaemia) is characterized by definite changes in the pattern of the proteins synthesized, with an increase in quantity of a set of proteins that we identified as heat-shock proteins [1–3]. These results were soon confirmed [4]. The enormous amount of data on the nature, mechanism of induction and functions of heat-shock proteins which are now more appropriately considered as stress proteins, because they are induced by many different and seemingly unrelated stress-producing agents, have been recently summarized [5–8]. The best known and most studied of these proteins belongs to the family of hsp 70, which in the rat comprises three members. Reperfusion increases the expression of the hsc 73 gene, which is constitutive and decreases during ischaemia, and induces the expression of the hsp 70 gene, the inducible member of the family; the third member, the glucose-regulated grp 78 genes, is induced much later, in relation to the onset of the acute-phase response to surgical trauma. Run-on experiments have demonstrated that the increased expression of these genes is largely dependent on activation of transcription [9]. Comparative studies with rat livers stored for transplantation (cold ischaemia) and with transplanted organs have shown that the early stages of liver transplantation are characterized by a depressed capacity of expression of some genes, such as those for albumin, transferrin and $\beta$-actin, which are well expressed in post-ischaemic reperfused livers *in vivo*, without the reactive phenomenon of activation of stress genes, and of hsp 70 in particular [10]. Apparently, the events occurring in the ischaemic liver in situ, which are prevented during cold storage in appropriate media, play a prominent role in the activation of the hsp 70 gene. Indeed, further studies have shown that the expression of the hsp 70 gene appears only after a certain threshold of cell damage, is preceded by induction of c-fos and c-jun, but does not depend on ongoing protein synthesis, which can be suppressed by cycloheximide without abrogating the response. The binding of the specific heat-shock transcription factor (HSF) to the heat-shock consensus sequence HSE seems to start already during the late period of ischaemia, although the subsequent reperfusion amplifies the effect [11]. The level of haem oxygenase (HOx) mRNA, an indicator of oxidative stress [12], increases in the liver after reperfusion, but the oxidative stress caused by $CoCl_2$ treatment does not induce the expression of the hsp 70 gene: therefore, a model of chemically induced oxidative stress seems to be unable to induce the hsp 70 gene expression with the same characteristics of heat shock or ischaemia reperfusion [11]. Reactive oxygen species are certainly generated during post-ischaemic reperfusion [13], and are likely to cause oxidative stress, but the facts described above suggest a more complex relationship between oxidative stress and induction of the main hsp gene. To define these relationships better, we have now studied the effects on rat liver gene expression of some drugs, known to induce oxidative stress by different mechanisms [14]. We have used: (i) buthionine sulphoximine (BSO), which blocks selectively the synthesis of glutathione by inhibiting $\gamma$-glutamylcysteine synthetase [15]; (ii) Phorone, which strongly decreases the concentration of glutathione through the action of glutathione S-transferase [16,17]; (iii) nitrofurantoin (NF), which causes the formation of $O_2^{-\cdot}$ and $H_2O_2$ by redox cycling [18,19] and finally lowers glutathione concentration [20]. Steady-state levels of mRNAs for hsp 70, HOx, c-fos, c-jun and metallothionein I (MT-I), superoxide dismutase (either Cu-Zn SOD or

Abbreviations used: BSO, buthionine sulphoximine; NF, nitrofurantoin; HOx, haem oxygenase; MT-I, metallothionein I; ODC ornithine decarboxylase; HSF, heat-shock factor; AP-1, activator protein 1; AP-2, activator protein 2; NFkB, nuclear factor kB; DOC, deoxycholate; EMSA, electrophoretic mobility shift analysis; TRE, 'TPA' (phorbol 12-myristate 13-acetate)-responsive element.

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Mn SOD) and ornithine decarboxylase (ODC), have been measured, and some run-on experiments, to assess transcription activation, have also been performed. The binding reaction between the transcription factors HSF, activator proteins 1 and 2 (AP-1, AP-2) and nuclear factor kB (NFkB) and the oligonucleotides analogous to their respective consensus sequences have been investigated by electrophoretic mobility shift analysis (EMSA). Oxidative stress induced by the chemicals used in this investigation induces or increases the expression of HOx and hsc 73, but does not induce hsp 70. NFkB and AP-2, but not AP-1 and HSF transcription factors, seem to be involved in the response.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (weighing 250–300 g) were used throughout, and were housed, fed and handled in compliance with the prescriptions for the care and use of laboratory animals.

### Chemically induced oxidative stress

Rats were injected subcutaneously with a single dose of $CoCl_2$ (300 $\mu$mol/100 g body wt.) intermediate between those used by Maines and Kappas [14] and by Tomaro et al. [21]. Phorone (diisopropylidene acetone), dissolved in sunflower oil, was injected intraperitoneally at a dose of 30 mg/100 g body wt. [22], which is known to increase the enzymic activity of HOx. BSO was injected intraperitoneally at a dose of 90 mg/100 g body wt. [23]: two injections were performed, one at zero time and a second one after 90 min: time of treatment was calculated from the time of the first injection.

NF, dissolved in DMSO, was injected intraperitoneally at the dose of 10 mg/100 g body wt. [18,24].

Heat shock and ischaemia–reperfusion, studied occasionally for comparative purposes were induced as previously described [11].

### Northern-blot analysis

RNA was extracted from total liver homogenate by a single-step method as previously described [9]: RNA concentration was determined spectrophotometrically, and then equal amounts (30 $\mu$g) of total RNA were electrophoresed under denaturing conditions. The rRNA concentration in each lane was estimated visually in the ethidium bromide-stained gels to confirm that each lane contained equal amounts of total RNA. RNA was transferred to Hybond C extra filters (Amersham International, Amersham, Bucks., U.K.) and hybridized with the various probes [9] labelled by nick translation by using a commercially available kit (Amersham). As an internal control, the same blot was probed with a $\beta$-actin probe. Post-hybridization washes were performed at 42 °C in 1 × SSC (150 mM NaCl/15 mM trisodium citrate, pH 7) containing 0.1 % SDS. Binding of the probes was detected by autoradiography.

### Run-on transcription analysis

Isolation of liver nuclei and run-on transcription *in vitro* were performed as described [10]. For the transcription reaction, $4 \times 10^7$ nuclei were resuspended in buffer (20 mM Tris/HCl, pH 7.4, 140 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 % glycerol, 1 mM ATP, GTP and CTP and 200 $\mu$Ci of [$^{32}$P]UTP, sp. radioactivity 800 Ci/mmol) and incubated at 26 °C for 15 min. The reaction mixture was diluted 10-fold with a buffer containing 10 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 50 mM $MgCl_2$ and 2 mM $CaCl_2$, and incubated with 50 $\mu$g/ml RNase-free DNase (Promega, Madison, WI, U.S.A.) for 30 min at 37 °C and then with 100 $\mu$g/ml Proteinase K for 30 min at 42 °C in the presence of 2 % SDS and 25 mM EDTA. After extraction with water-saturated phenol and chloroform, labelled RNA was precipitated twice with an equal volume of propan-2-ol. Samples containing equal amounts of radioactivity ($20 \times 10^6$ c.p.m.) were hybridized to an excess of different cDNA immobilized on nitrocellulose filters.

### Analysis of the mobility of DNA–protein complexes by gel electrophoresis

Nuclear extracts were prepared as described [11], in media containing antipain, chymostatin, pepstatin A, leupeptin (2 $\mu$g/ml each) and trypsin inhibitor (4 $\mu$g/ml). The oligonucleotides were synthesized by Primm (Milan, Italy). One strand was end-labelled with $T_4$ polynucleotide kinase and [$\gamma$-$^{32}$P]ATP and annealed to the complementary strand. The double-stranded sequence was purified by PAGE. Various amounts of extract were incubated in 25 $\mu$l of binding reaction mixture, containing 10 mM EDTA, 0.5 mM dithiothreitol, 0.5 $\mu$g of poly(dI-dC) and 0.1–0.5 ng of labelled double-stranded sequence. For competition experiments, an excess of specific and non-specific unlabelled double-stranded sequences (1–5 ng) was added to the binding mixture. After 20 min at 25 °C, samples were cooled on ice, 5 $\mu$l of dye solution (0.01 % Bromophenol Blue, 0.05 % xylene cyanol, 5 % Ficoll) was added, and samples were loaded on to a 5 % polyacrylamide gel in 45 mM Tris/HCl (pH 8.2)/1 mM EDTA/53.4 mM boric acid. Gels were run at 5 °C for 2 h at 20 mA, dried and autoradiographed at −70 °C with Kodak XAR film.

### Preparation of cytoplasmic extracts

Liver cytoplasmic extracts of control and Phorone-treated rats were prepared as follows. The supernatant of the pelleted nuclei, prepared for run-on transcription [10], was centrifuged to 100000 *g* for 1 h and then treated with $(NH_4)_2SO_4$ (300 mg/ml): the precipitate was resuspended and dialysed as described for the nuclear extracts [25].

### Oligonucleotides for EMSA

Oligonucleotides of the following base sequences were used:

HSE, CTAGAACGTTCTAGAAGCTTCGAG [26]

NFkB site, GGATCCTCAACAGAGGGGACTTTCCGA-GGCCA [25]

TRE ['TPA' (phorbol 12-myristate 13-acetate)-responsive element], CTAGTGATGAGTCAGCCGGATC [27]

AP-2, GATCGAACTGACCGCCCGCGGCCCGT [28]

### Probes

The following probes were used, generously given by the scientists mentioned in parentheses: human pURHS70 cDNA for hsp 70 (J.R. Nevins, New York) [29]; rat pUHC1 clone for hsc 73 (H. Pelham, Cambridge) [30]; rat pRH01 cDNA for HOx (S. Shibahara, Sendai) [31]; pMKMT1 cDNA for MT1 (A. Leone, Napoli) [32]; murine pc-fos-3 clone, mouse AH 119 for c-jun (F. Colotta, Milano) [33,34] and cDNA for rat $\beta$-actin (S. Gaetani, Rome).

## RESULTS

The hsp 70 probe used in the present experiments reveals two mRNA species, of 3.7 and 3.15 kb respectively, which are strictly inducible and correspond to the stress protein hsp 70, plus one additional band of ~ 2.7 kb, which is the result of the cross-reaction of this probe with the cognate hsc 73 mRNA, constitutively expressed and slightly inducible by exposure to heat [9,35]. The lane on the right side of Figure 1 shows how heat-shock induces hsp 70 mRNA and represents a positive control of the effectiveness of our probe. The hsp 70 mRNA is never induced by any oxidative-stress-inducing agent studied in the present work (Figure 1): this observation confirms the impression gathered from preliminary experiments performed with $CoCl_2$ [11]. On the contrary, hybridization with the specific probe shows that the expression of hsc 73 increases, albeit to a different extent and with different kinetics, in all the conditions of oxidative stress.

The induction of HOx, which is considered as a general response to oxidant stress in mammalian cells, is in contrast with the lack of induction of the stress-related hsp 70 mRNA. HOx is expressed at a very low level in normal liver, and is induced by all the treatments (Figure 2): the induction of HOx seems to be the final result of all the oxidative-stress-inducing agents considered in the present experiments, but the intensity and time course of expression can be influenced by the particular mechanism of action of any single agent.

Our interest in the MT-I gene was based on the observations that metallothioneins may play a direct role in response to oxidative stress, by functioning as antioxidants ([36] and references therein). The MT-I gene is already well expressed in normal rat liver, and further induction varies with the different agents: it is very high, and increases from 3 to 6 h, with $CoCl_2$ and BSO, but is less conspicuous with NF. Phorone, on the contrary, seems to depress the expression below the normal constitutive level: apparently, the oxidative-stress-inducing agent most effective in inducing HOx seems to possess an opposite effect on the MT-I gene (Figure 2). The immediate early-response genes c-fos and c-jun are induced promptly during the reperfusion stress that follows temporary liver ischaemia [9,11], as well as after exposure to a number of DNA-damaging agents, including UV: indeed, increased expression of c-fos has been considered as a general stress response of the cell [37,38]. In our experiments c-jun is induced, with decreasing intensity, by Phorone, NF and BSO, with mRNA levels that are higher at 3 h of treatment and decline or disappear at 6 h. Treatment with $CoCl_2$ does not induce c-jun. A small induction of c-fos has been observed only 3 h after Phorone treatment (Figure 2): this is another remarkable difference in gene expression between reperfusion stress [9] and chemically induced oxidative stress not apparently associated with DNA damage. Finally, none of the agents used in the present investigation induces SOD or ODC genes in rat liver (results not shown).

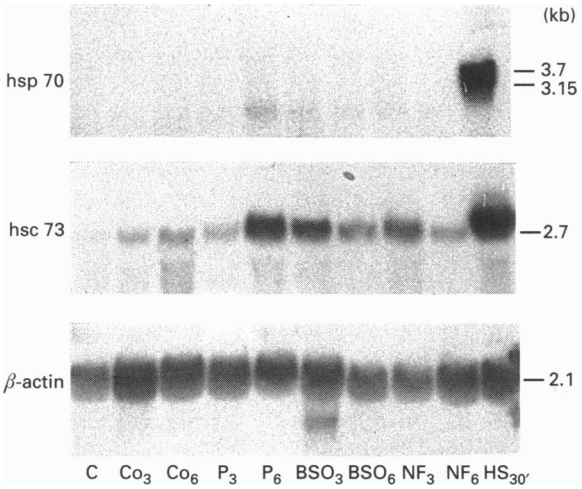


**Figure 1 Northern-blot analysis of hsp 70 and hsc 73 expression in rat liver subjected to oxidative stress**

All the data in this and the following Figures are from typical experiments with at least three different rats. Total RNA was extracted as described in the Materials and methods section. A 30 $\mu$g sample of each RNA was resolved by electrophoresis on a 1.2%-agarose gel, transferred to a nitrocellulose membrane, and hybridized with $^{32}$P-labelled hsp 70, hsc 73 and $\beta$-actin cDNA probes. Key: C, control rat; $Co_3$, $Co_6$, $P_3$, $P_6$, $BSO_3$, $BSO_6$, $NF_3$, $NF_6$, rats at different times (3 or 6 h) of treatment with $CoCl_2$, Phorone, BSO or NF respectively; $HS_{30'}$, rat made hyperthermic after 30 min of recovery at normal temperature. Values on the right indicate the size of the mRNAs.

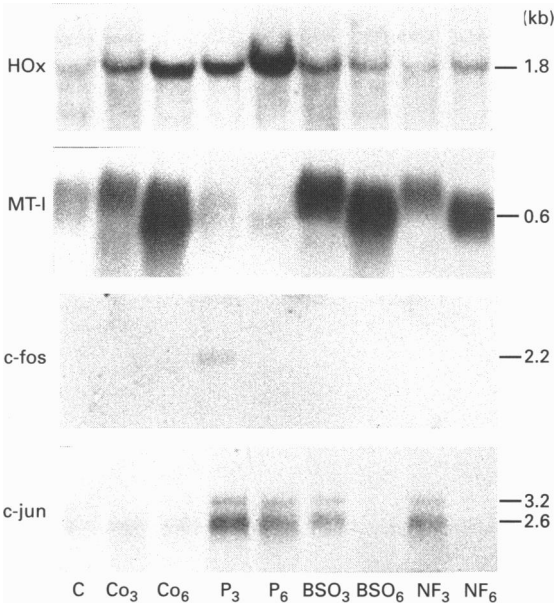


**Figure 2 Northern-blot analysis of HOx, MT-1, c-fos and c-jun expression in rat liver subjected to oxidative stress**

Total liver RNA was subjected to Northern-blot analysis as described in the Materials and methods section. A 30 $\mu$g sample of each RNA was resolved by electrophoresis on a 1.2%-agarose gel, transferred to a nitrocellulose membrane, and hybridized with $^{32}$P-labelled HOx, MT-I, c-fos and c-jun cDNA probes. Key as for Figure 1. Values on the right indicate the size of the mRNAs.

Run-on experiments with liver nuclei of Phorone-treated rats (Figure 3) demonstrate that the rise in the steady-state levels of hsc 73, fos, jun and HOx mRNA depends on transcriptional activation, though increased stability of the mRNA cannot be excluded as an additional mechanism: the unchanged rate of transcription of the $\beta$-actin gene indicates that increased transcription of hsc 73, fos, jun and HOx is selective and is not part of a general activation of the transcriptional processes.

Binding to the specific consensus sequence and activation of transcription factors, possibly associated with interaction with other DNA-binding proteins, constitutes the first step in the induction of gene expression. As a first approach to the under-

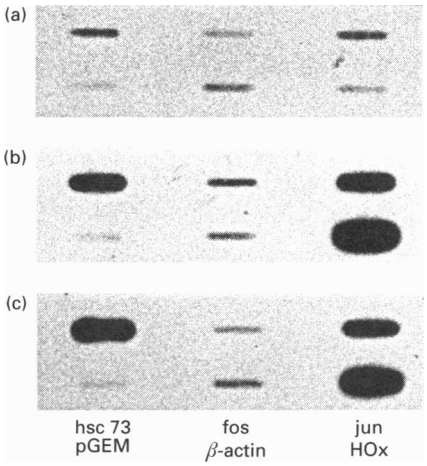


**Figure 3 Effect of Phorone treatment on hsc 73, jun, fos, β-actin and HOx mRNA transcription**

The $^{32}$P-labelled transcripts from isolated rat liver nuclei were hybridized with filter-bound immobilized plasmids containing cDNA for hsc 73, fos, jun, pGEM, β-actin and HOx as described in the Materials and methods section. $^{32}$P-labelled transcripts were obtained from liver nuclei from control rats (**a**) and from rats treated with Phorone and killed after 3 h (**b**) or 6 h (**c**). The autoradiogram was exposed for 1 week with intensifying screens. Positions of cDNAs in the panels are indicated by the scheme below (**c**).

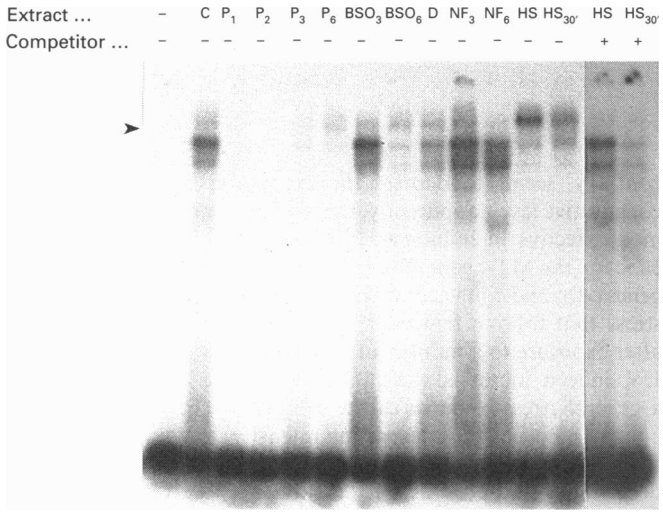


**Figure 4 Effect of oxidative stress son the HSF binding reaction**

Gel electrophoresis of HSE/protein complexes in liver nuclear extracts from control (C), Phorone (P)-, BSO- and NF-treated rats. Numbers represent times (h) of treatment. D, DMSO-treated rat (3 h); HS, heat-shocked rat; $HS_{30'}$, heat-shocked rat killed after 30 min of recovery at room temperature. Liver nuclear extracts (10 μg) were mixed with labelled HSE oligonucleotide (0.3 ng) in the absence (−) or presence (+) of 20-fold molar excess of unlabelled competitor oligonucleotide. Arrow at left of gel indicates the specific HSF–HSE-complex band.

standing of gene reprogramming during oxidative stress, we have performed EMSA experiments on liver extracts to study the binding of the transcription factors HSF, NFkB, AP-1 and AP-2 to oligonucleotides of the same base composition as their specific consensus sequences. HSF is the activator of heat-shock genes and is also active, directly or indirectly on the promoters of hsc 73, HOx and MT-I [39–41]. NFkB, a mediator of inducible gene transcription, is known to undergo redox regulation [42,43]:

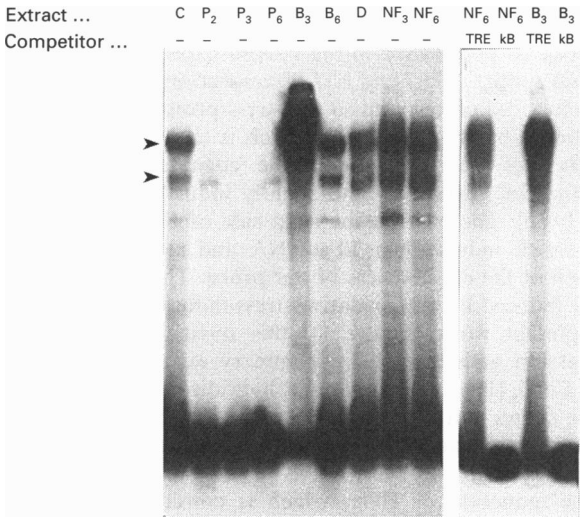


**Figure 5 Effect of oxidative-stress on the NFkB binding reaction**

Gel electrophoresis of NFkB-motif/protein complexes in liver nuclear extracts from: C, control rat; $P_2$, $P_3$, $P_6$, rats treated with Phorone and killed 2, 3 or 6 h later; $B_3$, $B_6$, rats treated with BSO and killed 3 or 6 h later; D, DMSO-treated rats (3 h); $NF_3$, $NF_6$, rats treated with NF and killed 3 or 6 h later. Liver nuclear extracts (5 μg) were mixed with labelled NFkB in the absence (−) or in the presence of unrelated oligonucleotide TRE or specific oligonucleotide kB-motif (kB). Arrows at left of the gel indicate the specific NFkB–NFkB motif-complex bands.

NFkB binding activity has been demonstrated in highly purified hepatocytes and appears to be inducible both *in vivo* and *in vitro*, thus supporting a role for NFkB in hepatocyte gene regulation [44]. AP-1, a multiprotein complex able to alter gene expression in response to many different agents [45], seems to be regulated by redox mechanisms [46]. AP-2, a regulator of developmentally regulated genes [47], is associated with NFkB in many cellular responses [48].

Among several bands observed in nuclear extracts from control liver, one is specifically increased by heat-shock (Figure 4): the specificity of this complex is demonstrated by the fact that it is completely competed by a 10-fold excess of unlabelled nucleotide. The appearance of this new band indicates that binding between the HSF, present in the nuclear extract, and the HSE-like oligonucleotide has occurred: the binding is more pronounced at the end of exposure to heat, and decreases during the recovery period at room temperature. The response was expected and is similar to that elicited by ischaemia–reperfusion [11]. Specific HSF–HSE binding is never observed in the other treatments studied in the present paper: the meaning of unspecific bands, uncompeted by the unlabelled nucleotide, cannot be defined, but, in view of the results with other transcription factors described below, it is worthwhile noting that these bands do not appear in the samples from Phorone-treated animals.

The experiments performed with AP-1 oligonucleotide give similar negative results (not shown). NFkB present in the normal rat liver binds to the appropriate consensus sequence, giving two distinct bands, in agreement with data from the literature [49] (Figure 5). Treatment with Phorone completely suppresses the binding; NFkB activity is markedly induced by BSO, in particular 3 h after treatment, and is consistently induced also by NF; the specificity of the response is apparent from the competition experiments with an excess of an unlabelled kB oligonucleotide, which completely suppresses the shifting, whereas an unrelated oligonucleotide of the sequence corresponding to TRE is

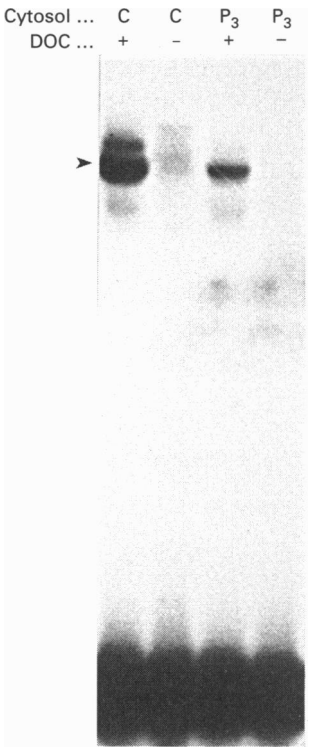


**Figure 6 Effect of DOC treatment on NFkB binding**

Gel electrophoresis of NFkB-motif/protein complexes in DOC-treated (+) or untreated (−) rat liver cytoplasmic extracts. Key: C, control rat; $P_3$, rat treated with Phorone and killed 3 h later. Mobility-shift binding-reaction mixture containing 50 $\mu$g of cytoplasmic protein extracts was incubated for 10 min on ice before addition of 0.4% DOC and incubation for an additional 10 min. The sample was adjusted to a final concentration of 1.2% Nonidet P-40 before electrophoresis. Arrow at left of gel indicates the specific NFkB–NFkB motif-complex band.

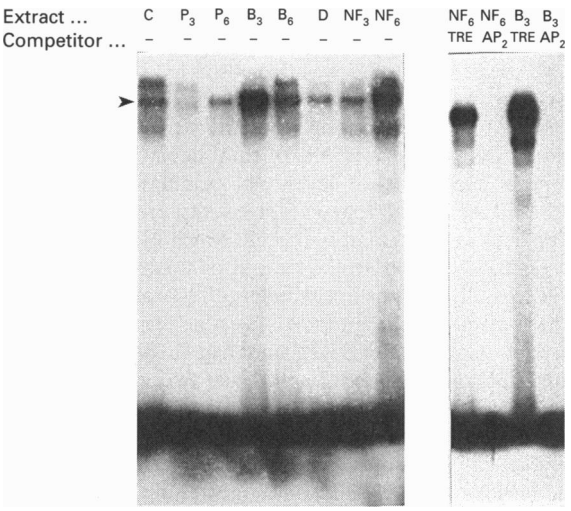


**Figure 7 Effect of oxidative stress on the AP-2 binding reaction**

Gel electrophoresis of AP-2-motif/protein complexes in liver nuclear extracts from: C, control rat; $P_3$, $P_6$, rats treated with Phorone and killed 3 or 6 h later; $B_3$, $B_6$, rats treated with BSO and killed 3 or 6 h later; D: DMSO-treated rats (3 h); $NF_3$, $NF_6$, rats treated with NF and killed 3 or 6 h later. Liver nuclear extracts (5 $\mu$g) were mixed with labelled AP-2 motif in the absence (−) or in the presence of unrelated oligonucleotide TRE or specific oligonucleotide AP-2 motif ($AP_2$). Arrow at left of gel indicates the specific AP-2–AP-2 motif-complex band.

ineffective. Taken together, these results indicate that NFkB might play a role in the effects of some oxidative-stress-inducing agents, with the exception of Phorone, which also interferes with the constitutive binding capacity of this transcription factor. Experiments of activation of NFkB *in vitro* were done in an attempt to understand the data obtained with phorone, but gave results of uncertain interpretation. NFkB is usually maintained in an inactive state in the cytosol by its association with the specific inhibitor IkB: *in vitro*, treatment with deoxycholate (DOC) releases NFkB, which becomes available for the binding reaction with the specific oligonucleotide (Figure 6). Treatment with DOC of cytosol from Phorone-treated rats releases only a decreased amount of the factor (which could be present either in lower than normal amount or in a state that prevents its dissociation from the inhibitor, or for any other reason), which is therefore not fully available for binding. Alternatively, this decreased activation could mean that NFkB has already been activated in Phorone-treated rats, but could have been destroyed or prevented from reaching its site of activity inside the nucleus.

EMSA performed with the AP-2 consensus sequence gives essentially the same results as NFkB (Figure 7): Phorone suppresses the signal, treatment with BSO activates the AP-2 binding, although less than in the experiments with NFkB, and NF shows an activation which, in the case of AP-2, is higher after 6 h than after 3 h of treatment. The experiments with unlabelled nucleotides demonstrate the specificity of shifting.

## DISCUSSION

Of the two genes of the hsp 70 family investigated in the present research, it is the constitutively expressed hsc 73 which seems to be activated in chemically induced oxidative stress. Differences between inducers, and in particular the levels of expression of hsc 73 reached after different times of treatment, are most probably dependent on how promptly each agent causes oxidative stress in the liver of the living rat. While this paper was in preparation, activation of the constitutive, rather than of the inducible, hsp 70 has been reported by menadione treatment of CHO cells [50], although the probe used by those authors could not properly discriminate between the two mRNAs. Previously, Bruce et al. [51] had shown no accumulation of hsp 70 mRNA or protein in NIH 3T3 cells exposed to menadione, although HSF was activated.

Our work extends these negative observations to other oxidative-stress-causing agents, acting in the intact rat, and identifies one member of the hsp 70 family which is responsive to oxidative stress, in association with HOx and MT-I genes. The response does not involve HSF, but occurs concurrently with the activation of two other transcription factors, such as NFkB and AP-2, which are likely to trigger the response. The expression of hsc 73, which is higher in fast-growing than in slow-growing transplantable hepatomas [35], but is also elevated in regenerating liver [52], is generally considered to be growth-related, as opposed to the stress-related hsp 70. Increased expression of hsc 73 in oxidative stress is not entirely surprising, however, as it is also found in post-ischaemic reperfused livers, where it is associated with induction of hsp 70. Factors common to oxidative stress and post-ischaemic reperfusion (e.g. generation of active oxygen species) could lead to increased expression of hsc 73, whereas additional circumstances peculiar to reperfusion (e.g. protein misfolding or denaturation) would be responsible for the induction of hsp 70. At variance with the agents tested in the present investigation, arsenite and diethylmaleate, inducers of a

member of the hsp 70 family in PAEC cells in an intracellular GSH-sensitive manner [53], can activate the binding of HSF. The level of intracellular GSH was decreased by 20 and 65% in the cells exposed to arsenite and diethylmaleate respectively, but the former activated HSF more potently than the latter [54], suggesting that the final effects of thiol depletion on HSF–HSE binding are dependent on additional modulatory factors.

It should be noted that increased expression of hsc 73 in dividing cells is associated with high levels of expression of the ODC gene [52], which are lacking in post-ischaemic reperfused livers [9], as well as in the livers undergoing oxidative stress which are the object of the present investigation. Other cases of differential induction of HOx and hsp 70 have been recently reported [55] and related to at least two distinct modes of regulation known for the rat HOx gene: induction mediated by heat-shock transcription factor and induction mediated by haem-responsive transcription factor [56]. The recent identification of several NFkB- and AP-2-like binding sites in the 5′ untranslated region of the human HOx gene [57] suggests a regulatory role also for these transcription factors, which are likely to play a role also in the increased expression of hsc 73 during oxidative stress. Although no kB and AP-2 sites have been as yet described in the promoter of hsc 73, and a direct mechanism cannot be surely asserted, an increasing number of examples of protein–protein associations between various types of transcriptional regulatory factors appear to determine the specificity of gene activation. Activation of NFkB and AP-2, which are both subject to redox regulation, could be involved in this complex interplay. The inhibition of binding observed in Phorone-treated rats is still open to many interpretations: most probably the extreme decrease in glutathione concentration, to about 5% of the normal liver content [17], induces oxidative stress at first, but can later prevent the binding to the DNA of the activated factor, which, in the case of NFkB, requires the integrity of a cysteine molecule [58] possibly compromised under extreme reducing conditions. Run-on experiments with liver nuclei of Phorone-treated rats demonstrated an increased transcription of hsc 73 and HOx: and we have also recently shown a distinct effect of Phorone on ferritin synthesis and gene transcription [59]. Therefore we think that the inhibition of transcription-factor binding, and the disappearance of non-specifically shifted bands in nuclear preparations of Phorone-treated rats, might depend on technical reasons and does not reflect the conditions occurring inside the cell.

In conclusion, several agents known to induce oxidative stress in the liver by different mechanisms of action are capable of increasing the expression of HOx, which is considered a constant feature of oxidative stress and is observed also in post-ischaemic reperfused livers. These same agents enhance the expression of the cognate hsc 73 gene, but do not induce the stress-related hsp 70 gene. Therefore the activation of hsp 70 by heat-shock and post-ischaemic reperfusion is not likely to depend on the oxidative-stress component present in the latter conditions, but may be triggered by a different kind of molecular damage acting as a signal. According to the present ideas, this could be identified in protein misfolding and/or denaturation [5]. This conclusion is reinforced by the fact that also the pattern of induction of the early-immediate response genes c-fos and c-jun observed during the chemically induced oxidative stress differs from that found in post-ischaemic reperfused livers [9].

The agents of oxidative stress studied in the present work do not activate the heat-shock factor HSF, involved in the response to heat-shock and post-ischaemic reperfusion. HSEs are present in the promoters of HOx and MT-I genes, but apparently are not the targets of the oxidative-stress-induced activation of these genes, which seems to occur concurrently with an activation of the transcription factors NFkB and AP-2. The same transcription factors are likely to be involved, most probably in co-operation with other factors [60] in the associated increased expression of the hsc 73 gene.

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