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Activation of transcription factors by drugs inducing oxidative stress in rat liver

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Abstract

Chemically induced oxidative stress of the liver associates with gene reprogramming and activation of some transcription factors (TFs), in particular nuclear factor-κB (NF-κB). We have now investigated other TFs, such as activator protein-1 (AP-1) and hypoxia inducible factor-1 (HIF-1) that we had shown to be activated in rat liver during heat shock, ischemia or post-ischemic reperfusion, and signal transducer and activator of transcription (STAT), CCAAT/enhancer binding protein (C/EBP) and hepatocyte nuclear factor-1 (HNF-1), which may be involved in the response of the liver to injury. The expression of target genes, containing consensus sequences for these TFs was assessed by Northern and Western blot analysis. The rats were treated with buthionine-sulfoximine, nitrofurantoin (NF) or phorone (Ph), which cause liver oxidative stress with different mechanisms. All these agents activated AP-1, known to depend on redox state, HIF-1, initially described as an hypoxia-responsive TF, and STAT3, generally connected to the response to cytokines. HNF-1 a constitutive TF associated with liver-specific gene expression was not affected. The composition of AP-1 was slightly different according to the drug used for treatment. The levels of the mRNAs for heme oxygenase-1 (HO-1), Aldolase A and α_1-acid glycoprotein as well as the corresponding proteins increased after the treatments, thus, indicating that the activation of the TFs was functional. These observations suggest that the treatment of rats with drugs inducing oxidative stress causes a broad spectrum of changes in gene expression with features common to stresses generally considered as separate entities.

Keywords: Liver; Oxidative stress; Gene expression; Transcription factors; HIF-1; AP-1

1. Introduction

Reprogramming of gene expression has emerged as an important mechanism used by cells to respond, and possibly to adapt, to adverse environmental changes or toxic substances. The first to be properly identified, and the best known of these responses is the so-called ''heat shock response'' caused by the increase of the temperature above the level of the normal temperature of growth of a particular type of cell or organism [1–3].

In addition to exposure to heat, other noxious agents or unfavorable conditions such as oxidative stress, can activate the transcription of genes, which are, therefore, more properly defined as stress genes, coding for stress proteins [4,5].

The first step in transcription depends on the activity of specific protein factors known as TFs. Although single TFs can play a dominant role in determining the expression of certain genes, transcriptional activation generally involves the synergistic action of more than one specific TF, in addition to those of the basic transcription machinery. For this reason in previous papers on ischemia-reperfusion and heat shock we have extended our study to a broad spectrum of TFs, such as heat shock factor (HSF), NF-κB, HIF-1, AP-1, AP-2, activator of TF/c-AMP responsive element binding (ATF/CREB) and HNF-1 [6] and to some of their target genes.

The same experimental design, with some adjustments after the first results, has now been followed in the study of the livers of rats exposed to drugs known to induce oxidative stress where we had already investigated the

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Abbreviations: AP-1, activator protein-1; AP-2, activator protein-2; BSO, buthionine sulphoximine; C/EBP, CCAAT/enhancer binding protein; EMSA, electrophoretic mobility shift assay; HIF-1, hypoxia inducible factor-1; HNF-1, hepatocyte nuclear factor-1; HO-1, heme oxygenase-1; HRE, hypoxia-responsive element; HSE, heat shock element; HSF, heat shock transcription factor; hsp70, heat shock protein 70; NF, nitrofurantoin; NF-κB, nuclear factor-κB; Ph, phorone; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TFs, transcription factors; TRE, TPA responsive element.

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behavior of HSF and NF-κB [7]. The agents used, selected because of their different mechanism of action, were: (i) buthionine sulphoximine (BSO) which blocks selectively the synthesis of glutathione (GSH) by inhibiting γ-glutamylcysteine synthetase [8], (ii) Ph which strongly decreases the concentration of GSH through the action of GSH S-transferase [9,10], (iii) NF which causes the formation of superoxide anion and H_2O_2 by redox cycling and finally lowers GSH concentration [11,12].

With the livers of rats treated with these chemical agents we have now studied the DNA-binding capacity of HIF-1, AP-1, STAT, C/EBP and HNF-1 TFs by electrophoretic mobility shift analysis. The expression of some genes, which contain in their promoters consensus sequences for these TFs, has been assessed by the steady-state level of their mRNA and the amount of the corresponding proteins. The investigation of AP-1 is obvious in view of the dependence of this TF on redox conditions [13]. The analysis of HIF-1 and STAT seems a priori less justified because these TFs were initially associated with the response to lowered oxygen tension the former [14] and to cytokine the latter [15]. But more recently both JAK-STAT pathway [16] and HIF-1 [17] have been shown to be activated by reactive oxygen species (ROS). HNF-1 is a liver-specific TF that responds to various environmental changes and is a useful indicator of liver-specific transcriptional activity.

The observation that AP-1, which we had previously found to be activated by ischemia-reperfusion and heat shock, was also activated by chemically induced oxidative stress prompted the analysis of the component subunits. Indeed, AP-1 consists of Jun/Jun homodimers or Jun/Fos heterodimers that could vary in composition in relation to the stimulus applied and the signal transduction pathway used by the cell. Since AP-1 dimers composed of different Jun/Fos proteins have been shown to exert distinct transcriptional activities [18], the composition of AP-1 appears crucial for the biological response of the cell: therefore, we tried to identify the subunits involved in the response to oxidative stress by means of super-gel shift or immunodepletion experiments. Supergelshifts were also performed to identify the member(s) of STAT family involved in the response. Finally, determinations of ATP and reduced GSH in the livers of treated rats were done as a useful complement for the interpretation of our results and as help to understand possible differences in response to various agents.

2. Materials and methods

2.1. 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.

2.2. Treatment with chemicals inducing oxidative stress

BSO was injected intraperitoneally at a dose of 90 mg/100 g body wt.: 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 dimethylsulfoxide, was injected intraperitoneally at the dose of 10 mg/100 g body wt. Ph (diisopropylidene acetone), dissolved in sunflower oil, was injected intraperitoneally at a dose of 30 mg/100 g body wt.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from rat liver were prepared as described [6], in media containing antipain, chymostatin, pepstatin A, leupeptin (2 μg/mL each) and trypsin inhibitor (4 μg/mL). Protein concentration was determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as standard. Samples containing 5 μg of nuclear extracts from rat liver were preincubated in 10 mmol/L Tris pH 7.8, 50 mmol/L NaCl, 1 mmol/L EDTA, 5% (v/v) glycerol, 0.5 mmol/L dithiothreitol, 0.5 μg of poly (dI-dC) for 5 min at room temperature before the addition of 0.5 ng of labeled double-stranded sequence. The oligonucleotides were synthesized by Primm (Milan, Italy). One strand was end-labeled with T_4 polynucleotide kinase and γ-^{32}P-ATP (Amersham, Bucks, UK), and then annealed to the complementary strand. The double-stranded sequence was purified by PAGE. For competition experiments, an excess (50×) of specific unlabeled double-stranded sequences were added to the binding mixture. After 20 min at 25°, 5 μL of dye solution (0.01% bromophenol blue, 0.05% xilene cyanol, 5% ficoll) were added and samples were loaded onto a 5% polyacrylamide gel in TBE 1× (0.1 mol/L Tris pH 8, 0.09 mol/L boric acid, 1 mmol/L EDTA). Gels were run at 4° for 2 hr at 140 V, dried and autoradiographed at −80° with Kodak MR film.

For supershift assay, the nuclear extracts were preincubated in ice with 1 μg of antibodies for 90 min, before the incubation at 25° for 20 min with labeled oligonucleotide, and then electrophoresed as described above. All antibodies used for AP-1 and STAT supershift assay were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti c-jun, anti junB, anti junD, anti STAT1 and STAT3 goat polyclonal IgG are specific for the different subunits; anti Fos (K-25) goat polyclonal IgG recognizes all the members of Fos family (c-fos, fosB, fra-1, fra-2).

2.4. Oligonucleotides for EMSA

Oligonucleotides of the following base sequences were used and were synthesized by Primm (Milan, Italy): hypoxia-responsive element (HRE): 5′-GCCC**TACGTG-C**TGTCTCA-3′ [19], TPA responsive element (TRE): 5′-CTAGTGA**TGAGTCA**GCCGGATC-3′ [20], Sis inducible element (SIE): 5′-GATCCTCCAGCAT**TTCCCG-**

TAAATCCTCCAG-3′ [21], C/EBP: 5′-TGCAGA**TTGCGCAA**TCTGCA-3′ [22], HNF-1 site: 5′TTAACTCCCTTTA**GTTAATATTTGAC**AAGTTTG-3′ [23].

2.5. Northern blot analysis

Total cellular RNA was isolated as described [24] and equal amounts of RNA were electrophoresed under denaturing conditions. To confirm that each lane contained equal amounts of total RNA, the ribosomal RNA content in each lane was estimated in the ethidium bromide-stained gels. RNA was transferred to Hybond-N filters (Amersham, Bucks., UK) which were hybridized with the following kindly provided ^{32}P-labeled DNA probes: c-jun and c-fos (F. Colotta, Milan Italy), junD (M. Birrer, NIH, Bethesda), STAT3 (V. Poli, University of Dundee, Scotland), rat HO-1 (S. Shibahara, Sendai, Japan), human Aldolase A (from plasmid HFBCC77 obtained from ATTC) and α_1-acid glycoprotein.

2.6. Immunoblot assay

For the preparation of cytosolic cell lysates, liver samples were homogenized in 20 mM Tris–HCl pH 8, 200 mM LiCl, 1 mM EDTA, 0.5% NP-40 and protease inhibitors. Samples were then kept at 4° for 40 min and centrifuged at 13 *g* for 5 min. Aliquots of the supernatants containing equal amounts of proteins, determined using the Bio-Rad protein assay kit, were electrophoresed in 12% acrylamide–SDS gels and electroblotted to nitrocellulose membranes (Amersham, Bucks., UK). Membranes were saturated in 4 mM Tris–HCl, pH 7.6, 30 mM NaCl (TBS) containing 5% BSA and 0.1% Tween-80 and incubated with a 1:1 dilution of polyclonal antibodies against to human HO-1 or to rat Aldolase A from Santa Cruz Biotechnology (Santa Cruz, CA) or with 1:2 dilution of polyclonal antibody against α_1-acid glycoprotein (kindly provided by C. Libert).

After incubation with antigoat or antirabbit secondary antibody and extensive washing with TBS containing 0.1% Tween-80, HO-1, Aldolase A and α_1-acid glycoprotein were detected by chemiluminescence using an immunodetection kit (ECL Plus, Amersham, Bucks., UK) and quantitated by laser densitometry.

2.7. Densitometric analysis

The autoradiograms were quantified by densitometric analysis (OD/mm^2) using a LKB Image Master DTS system (Pharmacia Biotec., Hertfordshire, UK).

2.8. Glutathione levels

Liver was homogenized in 5% sulfosalicylic acid (100 mg/mL) maintained 5 min at 4° and centrifuged 5 min at 3000 rpm in an Eppendorf centrifuge in the cold. GSH content was measured spectrophotometrically on the supernatant according to the procedure of Griffith [25].

2.9. ATP content

Liver was homogenized in 6% TCA (100 mg/mL) and processed as described above. ATP content was measured on the supernatant by Sigma Diagnostics Adenosine 5′-triphosphate kit (Sigma, Saint Louis, MO).

3. Results

3.1. Analysis of the DNA-binding capability of TFs by EMSA

Results of a previous paper [26] and of some preliminary experiments indicate that dimethylsulfoxide or sunflower oil, vehicles of NF and Ph, respectively have no effect on DNA-binding of TFs: therefore, the results obtained in treated animals are compared to normal liver.

Although several other regulatory events are to be taken into account, binding to the specific consensus sequence and activation of TFs, possibly interacting with other DNA-binding proteins, constitutes the first step in the induction of gene expression. As a first approach to the definition of gene reprogramming during oxidative stress, we performed EMSA on nuclear liver extracts to study the binding of the TFs HIF-1, AP-1, STAT, C/EBP and HNF-1 to oligonucleotides of the same base composition as their specific consensus sequences. Binding of HIF-1 to a synthetic oligonucleotide containing HRE is induced 1 hr after treatment with BSO and increases later (Fig. 1). NF and Ph give a stronger activation, but the effect of Ph is less durable and is strongly reduced 4 hr after treatment. DNA-binding of AP-1, which appears in the gels as two distinct complexes, is also activated by all the agents, in particular NF and Ph, and the activation seems to be even stronger and more prompt than observed for HIF-1 with the same drugs. STAT shows the same behavior, with slight differences in the kinetics of activation for each drug. On the contrary, the DNA-binding of C/EBP, which occurs at an appreciable constitutive level in normal rat liver, is slightly decreased only in Ph-treated rats. Competitions with unlabelled nucleotides demonstrate the specificity of the results. The band that is not suppressed in the lane 4c of STAT is due to unspecific-complexes.

HNF-1 is a dominant liver regulatory protein constitutively active: it can also respond to environmental stimuli but does not seem to be responsive to oxidative stress-inducing agents, acting with different mechanisms. Under conditions of liver cell damage induced by ischemia, DNA-binding of HNF-1 was found to be reduced [6], but the present results indicate that the oxidative stress-inducing agents used in this investigation do not interfere with basic

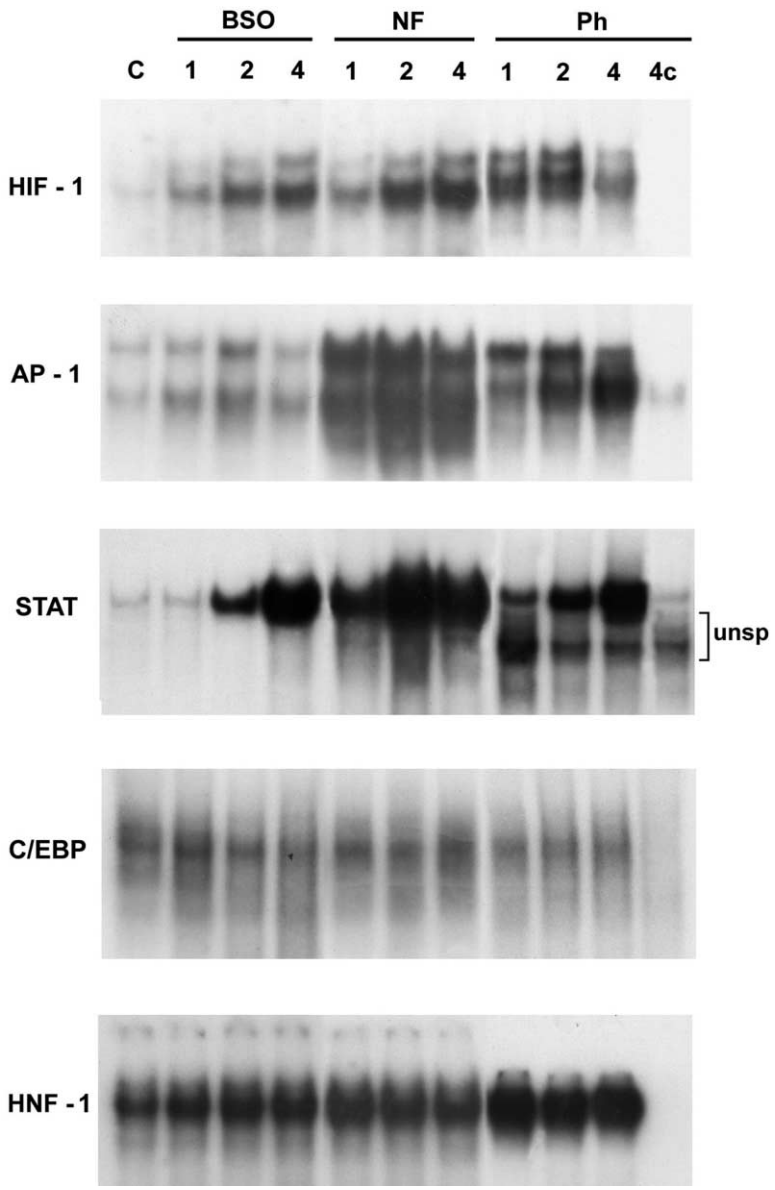

Fig. 1. Effect of BSO, NF or Ph treatment on the HIF-1, AP-1, STAT, C/EBP and HNF-1 DNA-binding reaction. EMSA of the aforementioned TFs in liver nuclear extracts (5 μg) from control rats (C) and from rats subjected to 1, 2, 4 hr of treatment with BSO, NF or Ph. For specific competitions liver nuclear extracts were mixed with an excess of unlabelled correspondent motifs (4c). The band (unsp) that is not suppressed by competition is an unspecific-complex. The results shown are representative of four independent experiments.

liver-specific gene expression. In summary, all the tested agents activate the DNA-binding capacity of HIF-1, AP-1 and STAT, though with different intensity and time-course: the DNA-binding of C/EBP, demonstrated with a canonical sequence common to all members of the family, is slightly decreased only in Ph-treated rats.

3.2. Supershift and Northern blot analysis of AP-1 subunits

Changes in the composition of a TF activated by different conditions, or at different times of treatment, could have a different impact on transcription and a distinct physiological significance. Antibody interference and supershift assay were performed to identify the components of AP-1 complexes in basal and activated conditions. We chose to study this TF because it is an heterodimer composed of different subunits and we restricted our investigation to the time where the activation of binding was more pronounced.

The analysis of AP-1 complexes was performed by using antisera specific for each member of Jun family, and an antiserum that recognizes all the members of Fos family. Using nuclear extracts from control and treated rats, the slower complex appears to contain c-jun and junD (supershift) (Fig. 2A). In Ph-treated rat liver preparations c-jun seems to be the major constituent of the slower band and supershift also occurs with Fos antibody. The response

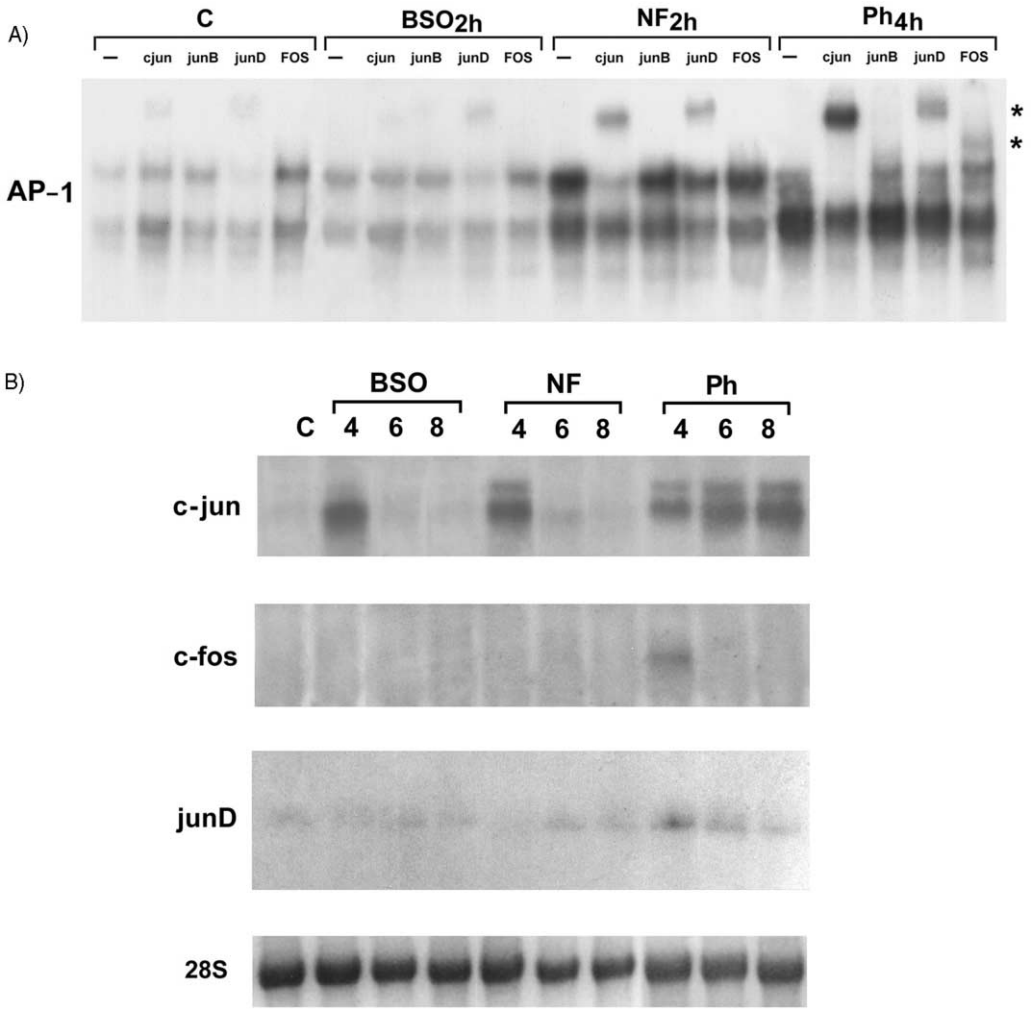

Fig. 2. Composition of AP-1 complexes in BSO, NF or Ph treatment. Panel A: supershift assays of nuclear extracts from control rat livers (C), or from rats subjected to BSO, NF or Ph treatment. Antibodies against c-jun, junB, junD and the Fos family subunits were used. Single asterisk: supershifted complexes. The autoradiograms shown are representative of four separate experiments. Panel B: Northern blot analysis of c-jun, c-fos and junD mRNAs levels. Filters with equal amounts of total liver RNA, as revealed by ethidium bromide fluorescence of 28S ribosomal RNA, were hybridized with probes for c-jun, c-fos and junD as indicated in Section 2. RNA was isolated from the liver of control rats (C), or from livers of rats subjected to 4, 6, 8 hr of BSO, NF or Ph treatment. The autoradiograms shown are representative of four independent experiments.

to Ph seems to be not only more marked but also qualitatively distinct.

Northern blot analysis of total liver RNA shows that c-jun mRNA is expressed as a peak at 4 hr with BSO and NF treatments and more persistently after Ph (Fig. 2B), while c-fos and junD are expressed only by Ph treatment.

On the whole the results of the Northern blots are the same but less pronounced than those obtained with the supershift analysis, which seems to be more sensitive. The most intense response is shown by c-jun, in agreement with the existence of a positive feedback between AP-1 and c-jun.

3.3. Supershift of STAT complexes and Northern blot analysis of STAT subunits

The analysis of STAT complexes was performed by using polyclonal antibodies anti STAT1 and STAT3. As shown in Fig. 3A, STAT3 seems to be the component of the STAT complexes in nuclear extracts from BSO, NF and Ph-treated rats. Northern blot analysis (Fig. 3B) shows the concomitant increase of the 5 kb mRNA for STAT3.

3.4. Expression of target genes

The promoter of every genes contains several sequences for different TFs that can potentially play a role in the induction of expression: however, activation of DNA-binding of these TFs does not necessarily imply that the gene will become activated. Determination of the steady-state levels of mRNAs for the genes containing these consensus sequences becomes essential.

HIF-1 activates many hypoxia-inducible genes through the consensus sequence HRE. The protein products of these genes increase O_2 availability, such as erythropoietin and promote metabolic adaptation to hypoxia, such as

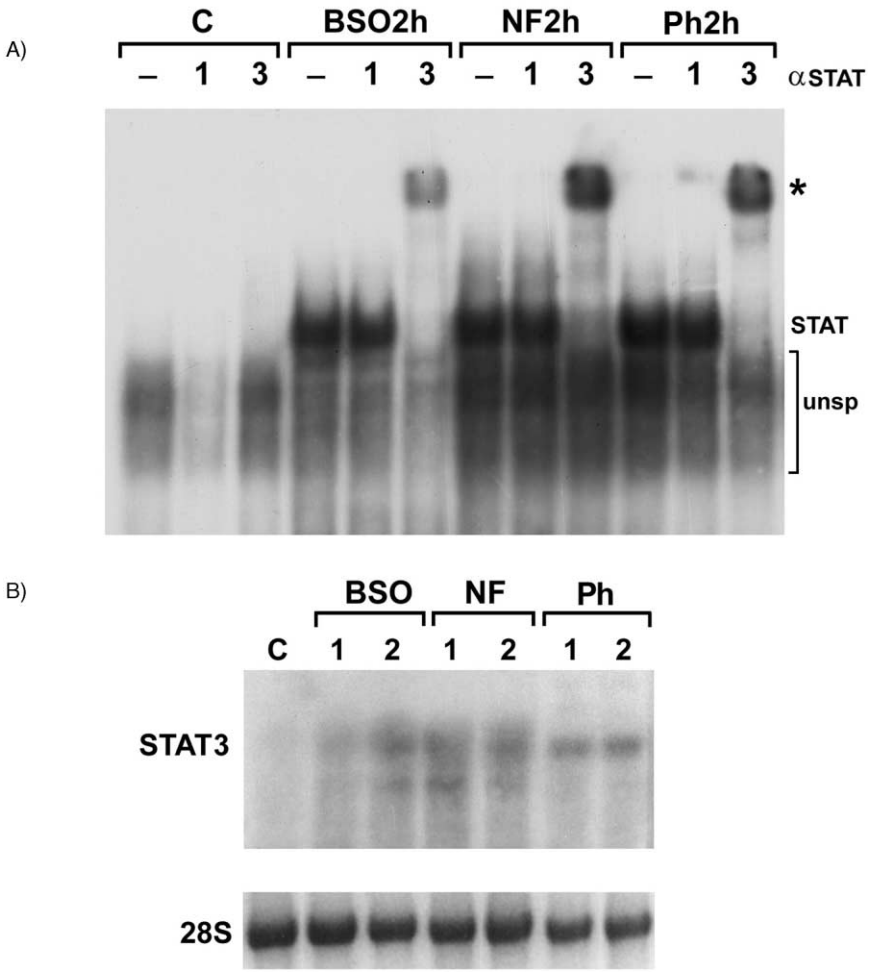

Fig. 3. Composition of STAT complexes in BSO, NF or Ph treatment. Panel A: supershift assays of nuclear extract from control rat liver (C), or rats subjected to BSO, NF or Ph treatment. Antibodies against STAT1 and STAT3 subunits were used. Single asterisk: supershifted complexes, (unsp): unspecific-complexes. The autoradiograms shown are representative of four separate experiments. Panel B: Northern blot analysis of STAT3 mRNA level. Filters with equal amounts of total liver RNA, as revealed by ethidium bromide fluorescence of 28S ribosomal RNA, were hybridized with the probe for STAT3 as indicated in Section 2. RNA was isolated from livers of control rats (C), or from livers of rats subjected to 1 or 2 hr of BSO, NF or Ph treatment. The autoradiograms shown are representative of four independent experiments.

glucose transporters and glycolytic enzymes. HRE, HSE and TRE are also present in the promoter of HO-1, a typical stress-inducible gene.

All the oxidative stress-causing agents used in our experiments increase the steady-state level of HO-1 mRNA, albeit with different time-course and intensity (Fig. 4A). Ph and NF give a prompt increase; Ph is the most potent; BSO acts more slowly and gives a less pronounced response. The same observations hold for the glycolytic enzyme Aldolase A. Notably, there is lack of response of erythropoietin (not shown) which suggests the existence of alternative pathways of activation between HIF-1 DNA-binding and gene expression. Differential responses of expression of glycolytic enzymes and erythropoietin have been recently reported [28]. The steady-state levels of α_1-acid glycoprotein mRNA increases in all the treatments, with a progressive trend for BSO and NF and a substantial constancy in time for Ph. The concomitant increase in the amount of the proteins, detected by Western blotting (Fig. 4B) indicates that these mRNAs are normally translated. The enhanced levels of α_1-acid glycoprotein are in agreement with the activation of STAT and AP-1, but the increase in the amount of mRNA and protein for what is considered an acute phase reactant, rather than a stress protein, suggests that clear-cut distinctions between different responses of the liver to distinct adverse conditions are untenable. The induction of α_1-acid glycoprotein and of other acute phase proteins in lines of hepatoma cells subjected to hypoxia [28], reinforces this concept.

3.5. GSH and ATP content

GSH content (Fig. 5A) decreases after all the treatments with kinetics compatible with the mechanisms of action of the different drugs. Ph, which is a GSH depletor, acts very

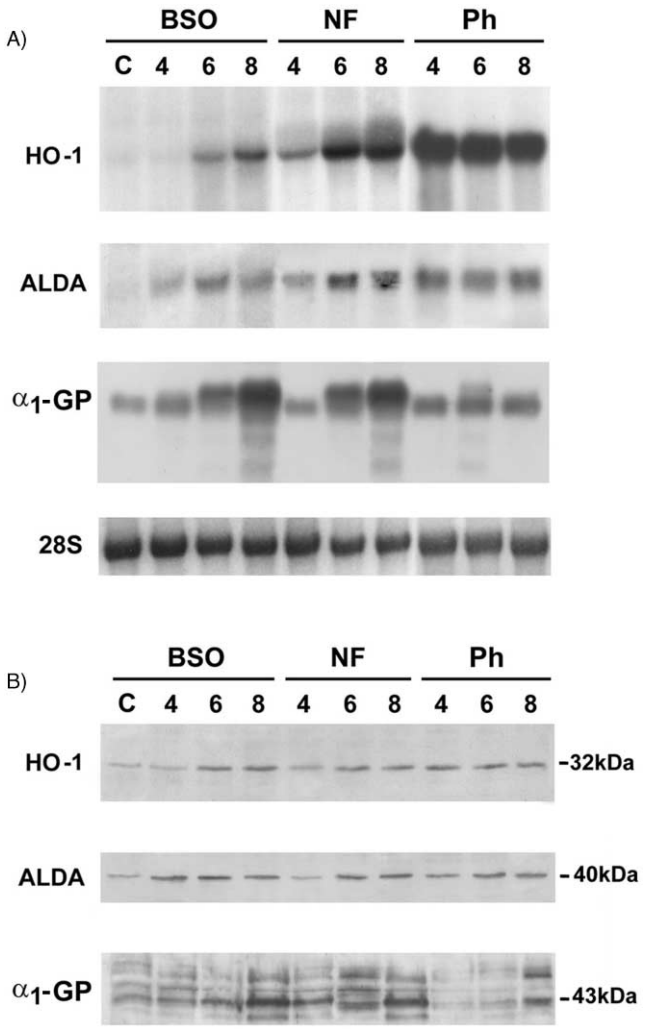

Fig. 4. Northern and Western blot analysis of HO-1, Aldolase A, and α_1-acid glycoprotein. Panel A: Northern blots. Filters with equal amounts of total liver RNA, as revealed by ethidium bromide fluorescence of 28S ribosomal RNA, were hybridized with probes for HO-1, Aldolase A (ALDA), α_1-acid glycoprotein (α_1-GP) as indicated in Section 2. RNA was isolated from the liver of control rats (C), or from livers of rats subjected to 4, 6, 8 hr of BSO, NF or Ph treatment. The autoradiograms shown are representative of four independent experiments. Panel B: Western blots. Extracts containing cytosolic proteins were prepared from control (C) or from livers of rats subjected to 4, 6, 8 hr of BSO, NF or Ph treatment. Equal amounts of proteins were processed as described in Section 2 and the HO-1, Aldolase A (ALDA), α_1-acid glycoprotein (α_1-GP) corresponding bands were visualized by chemiluminescence. The results shown are representative of three independent experiments.

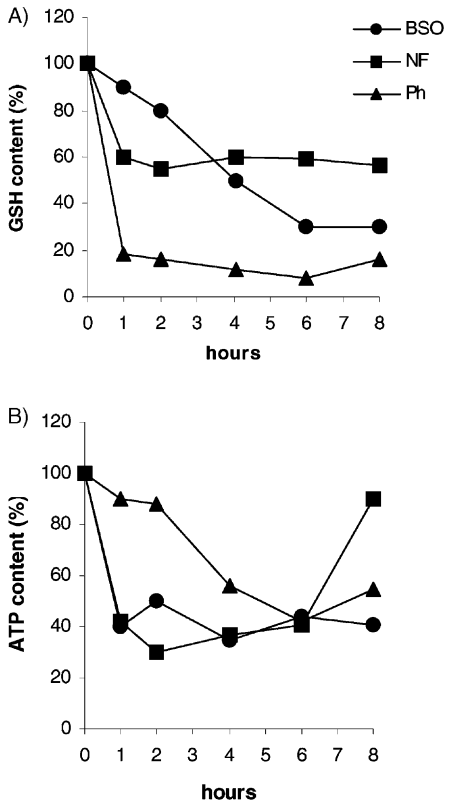

Fig. 5. Effect of BSO, NF or Ph treatment on hepatic ATP and GSH content. GSH (Panel A) and ATP (Panel B) content were determined spectrophotometrically as described. All values represent means of at least four separate experiments.

quickly and reduces GSH content to an extremely low level for a long time; BSO and NF cause important but less marked decreases. The situation is in part inversed for ATP content (Fig. 5B), which is promptly reduced by BSO and NF, and more slowly by Ph. After 6 hr of treatment the decrease is essentially the same with all the drugs, but NF-treated livers show a trend towards recovery in the next 2 hr. These results indicate that the oxidative stress-inducing agents used in the present experiments cause a change in both redox- and energy-state of the liver.

4. Discussion

Exposure of cells to environmental stresses results in activation of several signal transduction pathways, that culminate in the induction or stimulation of specific genes. Very often this is achieved by increased transcription, supported by activated TFs. We have previously investigated the activation of heat shock- and oxidation-specific stress genes in the liver of rats during chemically induced oxidative stress and focused our attention on the TFs HSF, NF-κB and AP-2 [26]. The observation that other TFs such as HIF-1, AP-1 and HNF-1 are involved in gene reprogramming of the liver that accompanies heat shock and post-ischemic reperfusion [6], a condition for many aspects similar to oxidative stress, suggested the extension of the research to the liver of rats treated with drugs known to induce oxidative stress. We investigated HIF-1, typically responsive to hypoxia, AP-1 sensible to pro-oxidant and antioxidant conditions, STAT family induced by a wide variety of growth factors and cytokines, C/EBP responsive to inflammatory stimuli and HNF-1, a dominant liver regulatory protein that can also respond to environmental changes and is a useful indicator of liver-specific transcriptional activity. The indication of the processes in which these TFs are involved should not be taken too strictly,

because a remarkable but not unexpected feature of some of these TFs is the capacity to respond to a large variety of inducing conditions. In the definition of oxidative stress, emphasis has been given sometimes to the generation of increased amounts of ROS and sometimes to the decrease of the scavenging and buffering capacities against them. Therefore, we used drugs that possess different mechanisms of action. With some minor differences in time-course and intensity, these drugs activated the DNA-binding capacity of HIF-1, AP-1 and STAT, identified as STAT3 by supergelshift and Northern blot analysis, in the liver of treated rats. Activation of HIF-1 by oxidative stress is counterintuitive. In our previous work, the DNA-binding of HIF-1 could be seen during ischemia, which includes anoxia, but disappeared during reperfusion [6]. But activation of HIF-1 was also observed during heat shock, which is a pro-oxidant condition. The decrease of ATP demonstrated during chemically-induced oxidative stress and typical of anoxic tissues could indicate a damage to mitochondria, with an eventual condition of anoxia superimposed on a state of oxidative stress. The decrease in GSH, which is the most important intracellular reductant, is also likely to play a role in the effects observed in the present experiments. Conflicting results have been reported on the capacity of exogenous oxidizing (H_2O_2) or reducing agents (DTT) to activate or inhibit HIF-1 [29]. Complex mechanisms of activation, including the mediation of a heme-containing protein, certainly play a role, as indicated by the activation caused by divalent metals, such as $CoCl_2$, or iron chelators, such as desferoxiamine [30]. The mechanisms of action of the latter compounds have not been defined but are distinct from the pathways used by hypoxia ([27] and references therein). It is worth noting that $CoCl_2$ is a typical inducer of HO-1, which is considered as an indicator of oxidative stress [31]. We found induction of HIF-1 DNA-binding by heat shock, concomitant with TFs activity and gene expression associated with oxidative stress [6,26]. HIF-1 activity was recently reported in cultured cells treated with the organomercurial compound mersalyl [27]: however, this drug inhibited endogenous erythropoietin mRNA induced by hypoxia, $CoCl_2$ or desferoxiamine. Our results of the lack of expression of erythropoietin in the presence of activated DNA-binding of HIF-1 and of expression of two HIF-1 target genes HO-1 and Aldolase A agree with the above findings from the literature. All these complex and sometimes seemingly contradictory data, including our ones, can be better understood in the frame of the recently expressed concept of context-dependent transcriptional regulation [32]. The concept refers to instances in which the transcriptional properties of a particular factor are influenced either by its position relative to other factors bound to a given promoter or by the abundance of transcription cofactors in a given cell type: transcriptional activity can also be influenced by the cellular environment in which the assays are performed [32].

The activation of AP-1 by drugs inducing oxidative stress is easier to understand: its nature of secondary antioxidant-responsive TF [13], activatable during ischemia as well as during late times of reperfusion [6] explains the response to pro-oxidant (NF) and reducing (hypoxia) conditions. But it is not excluded that the activation by hypoxia could also be mediated by small amounts of ROS that have been reported to be generated during hypoxia [33–35].

In a heterodimeric TF the composition in subunits is possibly more relevant than the strength of response, due to the different transactivating capacity of the resultant complexes. From this point of view the response to Ph, which comprises activation of Fos family members, seems to be more complete and more similar to that obtained with post-ischemic reperfused and heat shocked livers [6].

STAT3 is another TF activated by the chemicals considered in the present investigation. The STAT proteins were identified in the last years as TFs critical in mediating virtually all cytokine driven signaling, but occasionally other routes of STAT activation have been reported [36]. Relevant to the present results is the fact that STAT3 is activated by H_2O_2 in many lines of fibroblasts and in epidermally derived tumor cells. The activation was found to be oxidant-specific and did not occur in response to superoxide or nitric oxide generating stimuli, but was also described after 5 hr of exposure to BSO [37]: earlier time-points were not investigated but from our results we suppose that the drug may act more quickly also on cultured cells. In the more complex model of the living animal not only inhibitors of GSH synthesis, but also primary GSH depletors, such as Ph, or generators of superoxide, such as NF, activate the DNA-binding of STAT3, which seems, therefore, a more general condition induced by oxidative stress. Members of the STAT family are activated concurrently with AP-1 by hyperoxia and cooperate to the induction of HO-1 gene [38].

In the liver and hepatic cells members of C/EBP family are activated in the acute response to inflammatory stimuli, including IL-6 and IFN-γ which are typical activators of STAT [39]. On the contrary, the DNA-binding of C/EBP is unchanged or possibly reduced in our treated rats, indicating the existence of alternative separate pathways of activation for STAT and C/EBP. The activation of the DNA-binding of HIF-1, AP-1 and STAT3 by BSO, NF and Ph is functional to gene expression: indeed, genes which contain consensus sequences specific for these TFs are activated in the liver of treated rats and appropriate analysis detects the corresponding mRNAs and proteins.

The concomitant activation of genes typical of oxidative stress and acute phase response fits in the frame of the reciprocal relationships between gene expression in various types of stress. After some conflicting opinions it was finally realized that the heat shock and acute phase genes could also be induced simultaneously [40] and that the two responses were not mutually exclusive [41]. The present

results show that the same applies to oxidative stress. Depending on the circumstances, the severity and the duration of treatment, combinations of the responses can be obtained. Most genes are regulated by mixing and matching different types of activators and repressors in a coordinated fashion which has been defined as ''orchestrated response'' [42] or ''signal orchestration'' [43]. Such a mechanism may be responsible, at least in part, for the functional pleiotropy that characterizes agents inducing different types of stress.

Acknowledgments

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