Modulation of the DNA binding activity of transcription factors CREP, NFκB and HSF by H_2O_2 and TNFα. Differences between in vivo and in vitro effects

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Abstract Human endothelial cells exposed to H_2O_2 showed reduced CREP DNA binding activity, enhanced HSF activation, and no induction of NFκB binding activity. Interestingly, H_2O_2 was able to induce NFκB subunit p65 translocation in the nucleus. In contrast, cells exposed to TNFα showed enhanced CREP binding activity, activation of NFκB and no induction of HSE-HSF complex. Addition of H_2O_2, diamide and iodoacetic acid to the binding reaction mixture markedly reduced the DNA binding ability of the three transcription factors. Thus free sulfhydryls were important in DNA binding activity of CREP, NFκB and HSF, and the lack of induction of NFκB by H_2O_2 in intact cells was likely caused by oxidation on a thiol, and not by a deficiency in the activation pathway.

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Key words: Aminotriazole; Buthionine sulfoximine; Dihydrorhodamine 123; Oxidant; Endothelium; Thioredoxin

1. Introduction

Reactive oxygen intermediates are generated under various physiological and pathological conditions, including inflammation, ischemia and reperfusion, sepsis, and UV irradiation. The vascular endothelium is one of the prime targets for excessive amounts of reactive oxygen. Thus, under oxidative conditions, endothelial cells change their phenotype and their barrier function is compromised, allowing cell adhesion and transmigration and favoring coagulation and thrombosis. These changes are cumulatively referred to as endothelial activation, and are essentially manifested by the induction of a range of pro-inflammatory genes including those encoding adhesion molecules, chemotactic cytokines and prothrombotic molecules. The control of this activation process takes place at many levels and includes modification and selective nuclear transport of transcription factors such as the cAMP response element (CRE) binding proteins, the nuclear factor NFκB and the heat shock factor HSF. The CRE binding proteins consist of a number of different protein factors such as CREB-327 (δ) and CREB-341 (α), CRE-BP1, CRE-BP2, CREM, CREB-2, and a family of activating transcription factors (for a review see [1]). We refer to all these proteins as CREP in this article. NFκB is composed of two polypeptide species of 50 kDa (p50) and 65 kDa (p65), and is found in an inactive form in the cytoplasm of most cell types in a heterotrimeric complex with the inhibitor IκB. Upon cell stimulation, the IκB is phosphorylated and dissociates from the heterotrimeric complex allowing the heterodimer p50-p65 to migrate to the nucleus and activate gene expression [2]. To date, two HSFs have been identified in mammalian cells, and in unstressed cells, these two HSFs exist in a non-DNA binding, cytoplasmic form. Activation of HSF into a DNA binding form is accompanied by oligomerization and nuclear translocation [3]. Because many of the inducible genes involved in endothelial activation contain elements in their promoter regions that can be recognized by the ubiquitous regulatory proteins CREP, NFκB and HSF, and since in the setting of inflammation, H_2O_2 is generated by the same cells as cytokines such as TNFα, it is of interest to know if exposure of endothelial cells to H_2O_2 and TNFα may actually lead to concerted activation of these major transcription factors.

2. Materials and methods

2.1. Chemicals and reagents

[γ-^{32}P]ATP (5000 Ci/mmol) and the DNA 5′ end-labeling kit were obtained from Amersham (Buckinghamshire, UK). Aminotriazole, buthionine sulfoximine, diamide, iodoacetic acid and protease inhibitors were from Sigma Chemical Co. (St. Louis, MO, USA). *E. coli* thioredoxin and thioredoxin reductase were purchased from IMCO Co. (Stockholm, Sweden). DHR123 was obtained from Molecular Probes Europe (Leiden, The Netherlands). NFκB p65 antiserum was purchased from Santa-Cruz Biotechnology (Heidelberg, Germany).

2.2. Cell culture

Human endothelial cells were obtained from umbilical cord veins and grown in RPMI 1640 supplemented with 25 mM HEPES, 10% fetal calf serum (FCS), 15 μg/ml endothelial cell growth supplement and 90 μg/ml heparin as previously described [4]. Second and third passage cells in monolayer culture were used for all experiments at confluence.

2.3. Experimental conditions

2.3.1. Exposure to hydrogen peroxide (H_2O_2). Cells were incubated at 37°C with different concentrations of H_2O_2 in Krebs Ringer pH 7.4 for the times indicated in the figures. Catalase was then added (100 U/ml) to remove the H_2O_2.

2.3.2. Exposure to TNFα. Cells were incubated with 100 and 500 U/ml of TNFα in RPMI containing 10% FCS at 37°C for 1–4 h.

2.3.3. Pretreatment with AT and BSO. Cells were incubated with 500 μM AT and 200 μM BSO in normal culture medium for 16h prior to exposure to H_2O_2 or TNFα.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and assayed for DNA binding activity by EMSA, as previously described [4]. The oligonucleotides used as probes were a double stranded CRE oligonucleotide (5′-CCGTGACGTCACCC-3′), NFκB (5′-AGAGGGGACTTTCCGA-

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Abbreviations: AT, aminotriazole; BSO, buthionine sulfoximine; CREP, cyclic AMP responsive element binding proteins; DHR123, dihydrorhodamine 123; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HUVEC, human umbilical vein endothelial cells; HSF, heat shock factor; NFκB, nuclear factor κB; ROI, reactive oxygen intermediates; TR, thioredoxin; TRX, thioredoxin reductase

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GA-3′) or HSE (5′-GCCTCGAATGTTCGCGAAGTT-3′) which were 5′ end-labeled using T_4 polynucleotide kinase and [γ-^{32}P]ATP. Where indicated, nuclear extracts were treated with the different agents for 15 min at room temperature before addition of the probe.

2.5. Gel electrophoresis and immunoblotting

Cytosolic and nuclear extracts for Western blot were prepared essentially as described [2], with the following modifications: lysis buffer contained 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml each of leupeptin, pepstatin A, antipain and aprotinin, and cell lysis was completed by pipetting through a P200 pipette-man tip. 25 μg protein of cytosolic extract and 15 μg protein of nuclear extract were separated in SDS-PAGE (10% acrylamide) and transferred to Immobilon-P membrane. NFκB p65 antiserum was used as primary antibody at a final concentration of 0.5 μg/ml, and the revelation of immunoblots was performed with an ECL kit from Amersham.

2.6. Clearance of H_2O_2 and measurement of intracellular ROI

The method used for quantitation of H_2O_2 remaining in the culture medium at different time points after treatment is based on the horseradish peroxidase-mediated oxidation of phenol red by H_2O_2 which results in the formation of a compound demonstrating increased absorbance at 610 nm [5]. To ascertain the contribution of intracellular catalase and glutathione/glutathione peroxidase in the observed H_2O_2 clearance, these measurements were repeated in AT/BSO pretreated cells. Estimation of intracellular ROI in living cells was performed using the cell permeable probe DHR123 [6]. Cells grown in a 96 well tissue culture plate were incubated with 5 μM DHR123 in RPMI without phenol red for 1 h at 37°C. The medium was then replaced with RPMI without (control) or with H_2O_2 or TNFα and the incubation continued for 1 h at 37°C. The media were removed, the cells were washed once with RPMI without phenol red, and the plates were read on a CytofluorII plate reader (excitation 490, emission 530).

2.7. Cytotoxicity of H_2O_2 and TNFα

Lactate dehydrogenase activity, used as a marker of cellular injury, was determined in the supernatants and cell extracts as previously described [4]. Cytotoxicity was expressed as the percentage of supernatant activity relative to total activity in the supernatant plus monolayer.

3. Results

3.1. Effects of H_2O_2 on CREP, NFκB and HSF binding activity in intact cells

HUVEC were incubated with increasing amounts of H_2O_2 (0.1–5 mM) for 20–240 min at 37°C. At the end of these incubation periods, nuclear proteins were extracted and CREP, NFκB and HSF binding activity tested by EMSA. The results in Fig. 1 revealed an inhibition of CREP binding activity, which was dose dependent and reversed by the addition of either 1 mM DTT or 80 μM TRX to the reaction mixture. Competition with cold CRE and TRE (tumor promoting agent responsive element oligonucleotide) confirmed the specificity of this binding. In contrast, H_2O_2 specifically induced HSF binding activity in a dose and time dependent manner, but did not activate NFκB.

3.2. Effects of TNFα on CREP, NFκB and HSF binding activity in intact cells

Intact cells were exposed to 100 and 500 U/ml of TNFα for 1 h at 37°C. As shown in Fig. 2, analysis of nuclear extracts from TNFα treated cells revealed a significant increase in CREP and NFκB binding activity, but no activation of

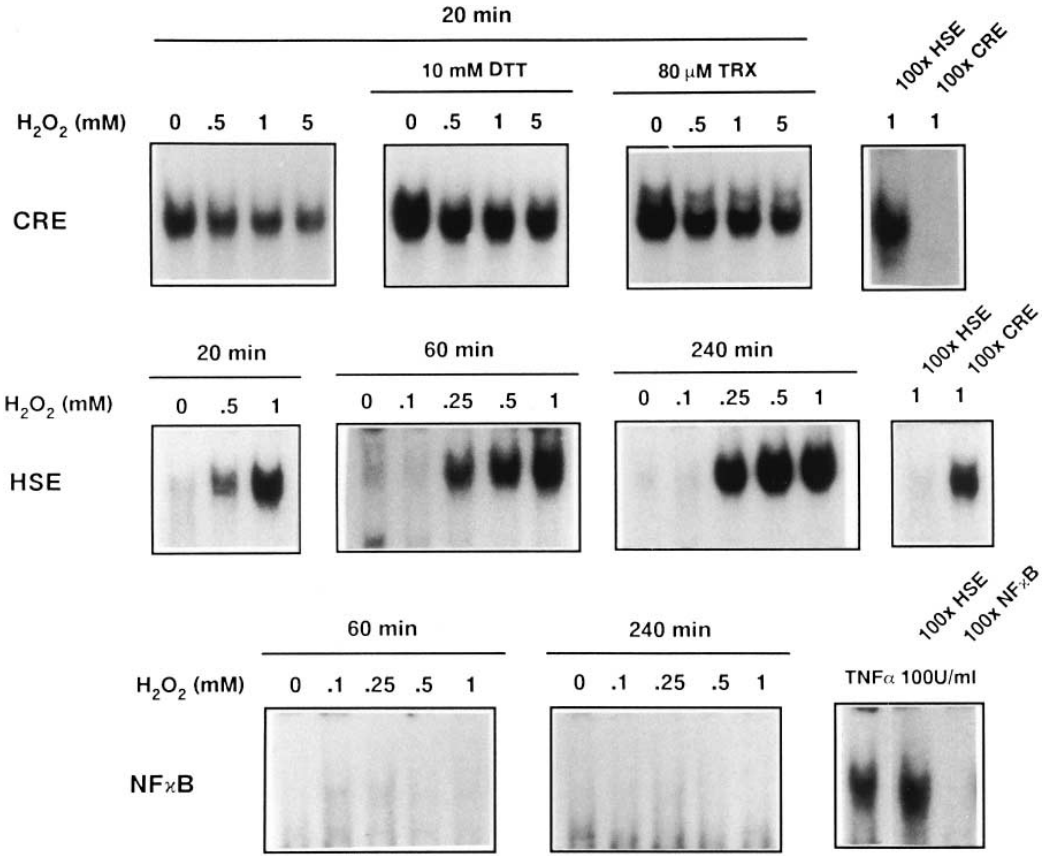


Fig. 1. H_2O_2 inhibits CREP binding activity, and activates HSF in a dose dependent manner, but does not activate NFκB in intact cells. Cells were exposed to increasing concentrations of H_2O_2 at 37°C for 20, 60 and 240 min. Addition of DTT and TRX to the binding reaction mixture partly reversed the inhibitory effects of H_2O_2 on CREBP binding activity. As expected, TNFα (100 U/ml, 1 h) does activate NFκB.

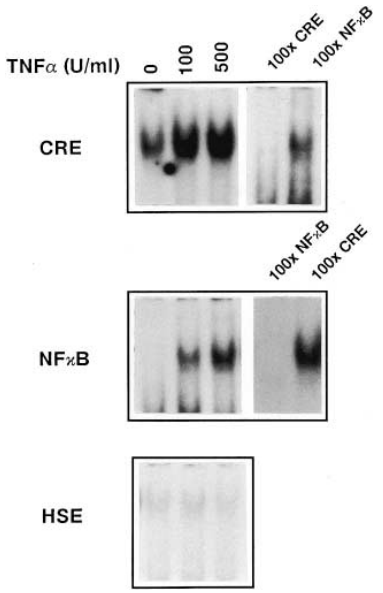


Fig. 2. TNFα (100 and 500 U/ml, 1 h) enhances CREP-CRE interaction, activates NFκB, but does not induce the formation of HSF-HSE complexes.

HSF. Competition with cold NFκB and CRE confirmed the specificity of these DNA binding activities.

3.3. *Effects of pretreatment with AT and BSO on the DNA binding activity of CREP, NFκB and HSF in intact cells exposed to H_2O_2 and TNF$_\alpha$*

To favor intracellular ROI accumulation, cells were pretreated with 500 μM AT and 200 μM BSO for 16 h prior to a challenge with H_2O_2 or TNFα. These treatments, which have been previously shown to completely inhibit catalase activity and glutathione synthesis in HUVEC [7], led to increased cellular H_2O_2 concentrations due to a decreased rate of H_2O_2 consumption (Fig. 6). As shown in Fig. 3, the effects of H_2O_2 and TNFα on CREBP and NFκB were essentially the same in AT/BSO pretreated and untreated cells, while the H_2O_2 induced HSF-HSE binding activity was accentuated in AT/BSO treated cells.

3.4. *H_2O_2, like TNFα, induces translocation of NFκB p65 in the nucleus*

NFκB activation is a multistep process that results in the translocation of a fraction of the p65-p50 heterodimer in the nucleus where it binds to DNA. We therefore investigated whether H_2O_2 was able to induce nuclear translocation of NFκB. To this end, cells were exposed to 250 μM and 1 mM H_2O_2 for 1 h and 4 h, or to 100 U/ml TNFα for 1 h, and the distribution of the p65 subunit in the nuclear and cytosolic fractions was analyzed by Western blotting. As seen in Fig. 4, a small fraction of the cellular content of p65 was translocated in the nucleus after 1 h treatment with H_2O_2. This translocation was more prominent by 4 h of H_2O_2 exposure. As expected, a significant amount of p65 was detectable in the nucleus by 1 h following TNFα addition.

3.5. *Reduced sulfhydryl groups in CREP, NFκB and HSF are required for DNA binding in vitro*

Nuclear extracts prepared from normal cells, H_2O_2 treated (500 μM, 1 h) or TNFα treated cells (100 U/ml, 1 h) were incubated with 1 mM H_2O_2 and the diazene carbonyl derivative diamide, two compounds which chemically catalyze the oxidation of free sulfhydryl groups, or IAA, a chemical agent that alkylates and thus blocks free sulfhydryl groups, before addition of the probe. As shown in Fig. 5, both chemical reagents almost completely abolished CREP and NFκB binding activity. The effects of H_2O_2 and diamide were fully reversible with the subsequent addition of the reducing agent DTT (10 mM) or the reducing system TRX-TR-NADPH (80 μM; 80 nM; 800 μM), whereas the effects of IAA were almost irreversible. Treatment of extracts with DTT and TRX alone significantly increased the strength of the bands, indicating that a fraction of the isolated CREP and NFκB in nuclear extracts existed in an oxidized state that could be reduced to achieve greater DNA binding. The oxidizing agents H_2O_2 and diamide also had an inhibitory effect on the HSF-HSE binding activity in vitro, which was completely reversed by DTT, whereas the alkylating agent IAA had no effect.

3.6. *Rate of H_2O_2 consumption and ROI levels in intact cells treated with H_2O_2 and TNFα*

The rate of consumption of H_2O_2 by endothelial cells was

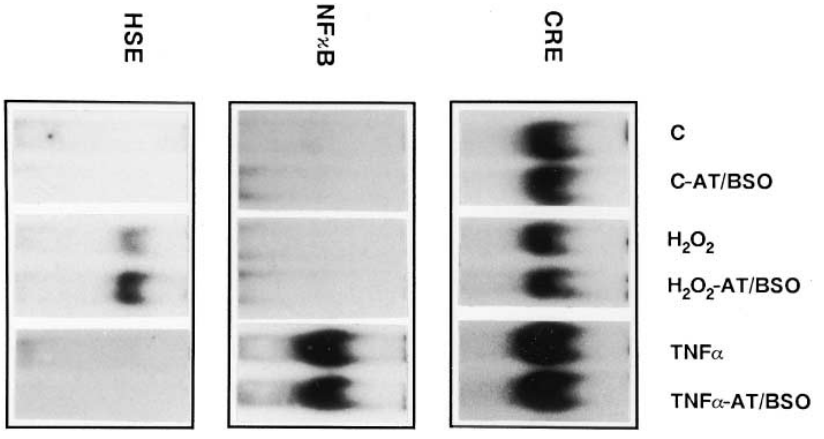


Fig. 3. Cells pretreated with AT and BSO show accentuated effects of H_2O_2 on HSF activation, but no change in the effects of H_2O_2 and TNFα on CREP and NFκB binding activity. To assess CRE-CREP interaction and NFκB activation, cells were exposed to 500 μM H_2O_2 for 20 min and 1 h, respectively. To assess HSF activation, cells were exposed to 250 μM H_2O_2 for 1 h. In separate experiments, cells were treated with 500 U/ml TNFα for 1 h.

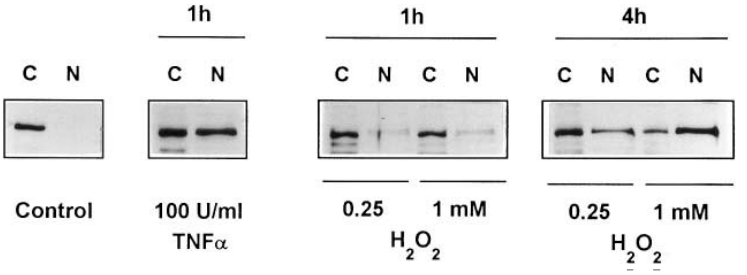


Fig. 4. TNFα and H_2O_2 induced nuclear translocation of NFκB p65 subunit. Immunoblot of p65 in cytosolic (C) and nuclear (N) extracts from untreated cells (control) and cells treated with 100 U/ml TNFα for 1 h or with 250 μM and 1 mM H_2O_2 for 1 h and 4 h.

evaluated by measuring the amount of H_2O_2 that remained in the culture medium at different time points after addition of H_2O_2. As shown in Fig. 6A, HUVECs were able to remove H_2O_2 since the H_2O_2 concentrations in the medium decreased rapidly with time. AT/BSO pretreated cells were still able to remove H_2O_2 from the extracellular medium, although at a slower rate. In the absence of cells, the H_2O_2 concentrations in the medium remained unchanged during a 1 h period, and decreased by 20–30% after 4 h at 37°C.

To determine the existence of a possible interconnection between the CREP, NFκB and HSF DNA binding activity and the cellular redox state, we estimated the intracellular ROI levels with the use of the cell permeable probe DHR123. Fig. 6B shows that exposure to 250 μM H_2O_2 led to a significant increase in intracellular ROI levels, which was enhanced in AT/BSO treated cells. TNFα (500 U/ml) did not stimulate ROI production since we failed to detect any enhanced rhodamine fluorescence as compared to untreated cells.

3.7. *Cytotoxicity of H_2O_2 and TNFα*

A short-term exposure to 5 mM H_2O_2 did not cause cytotoxicity: the percentage of LDH released by normal and treated cells was 1.8 ± 0.2 and 2.2 ± 0.4 after 20 min, 2.4 ± 0.8 and 2.5 ± 0.4 after 1 h of exposure ($n = 4$). Similarly, the percentage of LDH released after a 1 h treatment with 100 and 500 U/ml TNFα amounted to 2.2 ± 0.6 and 2.8 ± 1.0 ($n = 4$), respectively, which was not significantly different from normal cells (2.4 ± 0.8). Loss of cell viability was evident after 4 h incubation with H_2O_2, with LDH released in the

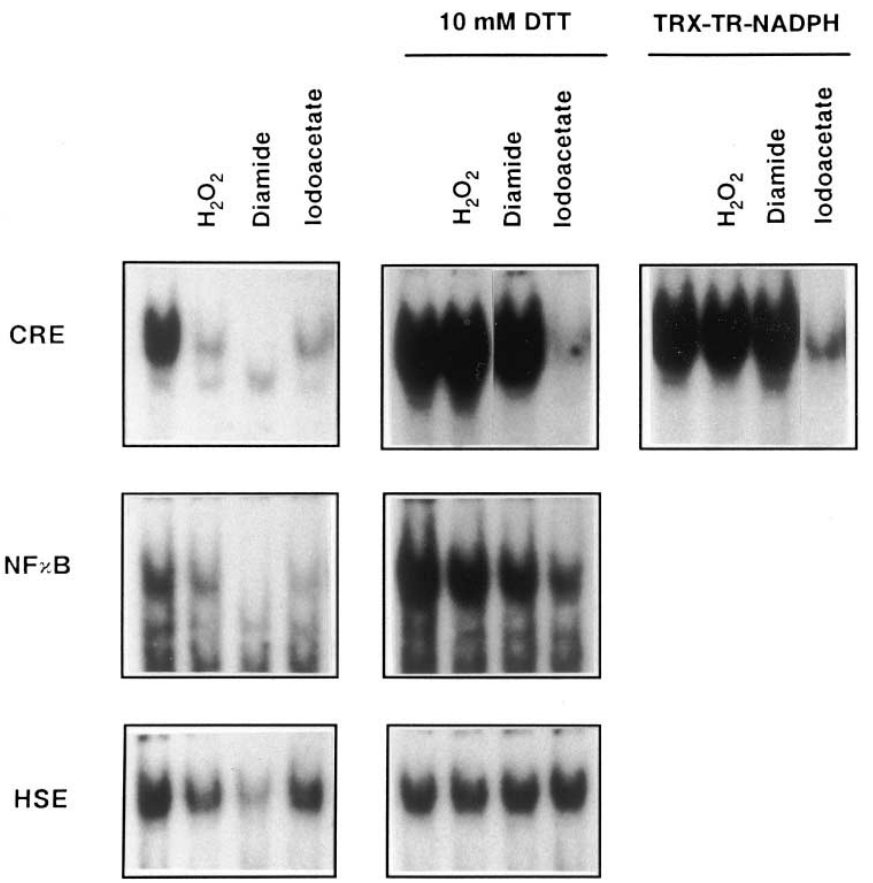


Fig. 5. Oxidation of sulfhydryls by H_2O_2 and diamide eliminates CREP, NFκB and HSF DNA binding activity in vitro. Alkylation of sulfhydryls by IAA also inhibits CREP and NFκB binding activity, but is ineffective on the HSF-HSE complex. The effects are fully reversed by addition of DTT or TRX-TR-NADPH.

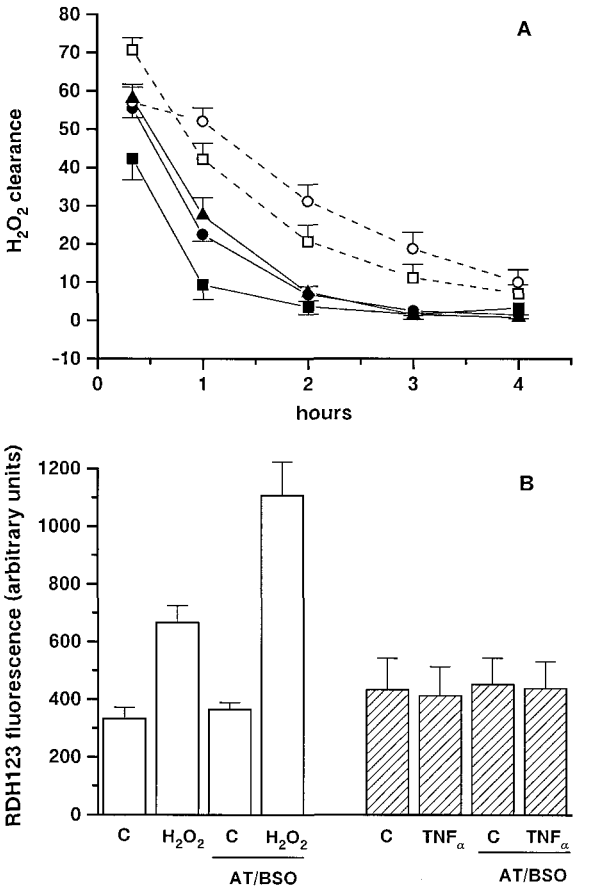


Fig. 6. A: Rate of H_2O_2 clearance by normal cells (solid lines) and AT/BSO pretreated cells (dashed lines). Results shown are the concentrations of H_2O_2 remaining in the medium at different time points after addition of H_2O_2 (250 μM, squares; 500 μM, circles; 1 mM, triangles), and expressed as % of the values in the absence of cells. B: Estimation of intracellular ROI levels following 1 h exposure to 250 μM H_2O_2 and 500 U/ml TNFα in normal cells and AT/BSO treated cells. ROI production is analyzed on the basis of oxidant induced DRH123 oxidation resulting in an increment of the mean rhodamine 123 fluorescence intensity expressed in arbitrary units. Data are means ± S.E.M. from four independent experiments.

medium increasing from a basal value of 4.2 ± 1.1% to 6.9 ± 1.1, 11.1 ± 2.2, 12.5 ± 3.1, 11.0 ± 2.5 and 31.4 ± 8 for cells exposed to 0.1, 0.25, 0.5, 1 and 5 mM H_2O_2, respectively ($n = 6$).

4. Discussion

We have demonstrated differential effects of H_2O_2 and TNFα on the DNA binding activity of CREP, NFκB and HSF in human endothelial cells. In intact cells, H_2O_2 and TNFα exerted quite opposite effects on the DNA binding activity of the three transcription factors, whereas in vitro modifications of free sulfhydryl(s), either by oxidizing or alkylating agents, markedly reduced their DNA binding ability.

Numerous studies have pointed to a role of ROI as a common second messenger system used by different stimuli to activate transcription factors. AP-1 and NFκB were the first eukaryotic factors shown to respond directly to oxidative stress [8,9]. Our observations confirmed important redox effects on two other ubiquitously expressed transcription factors, CRE and HSE binding proteins. These effects were, however, selective. Specifically, H_2O_2 dose dependently reduced the DNA binding activity of CRE binding proteins, while markedly inducing HSF. The two chemically distinct reductants DTT and TRX, when added to the binding reaction, were both effective, although partially, in reactivating CREP extracted from H_2O_2 exposed cells. This suggests that exposure of cells to a high dose of H_2O_2 likely led to inactivation by oxidation of the cysteine residue(s), and also to some irreversible loss of active CREP proteins, possibly a consequence of an enhanced instability of the oxidized proteins. The fact that H_2O_2 oppositely affected the DNA binding activity of CRE and HSE binding proteins suggests direct oxidation of cysteine residue(s) located in the DNA binding domain of CRE binding proteins, while the effect of H_2O_2 on HSF must lie upstream of HSF-DNA interactions, perhaps at the stage of oligomerization and/or nuclear translocation. A similar conclusion was reached by Jacquier-Sarlin and Polla, who proposed that HSF is under dual regulation: H_2O_2 favors HSF nuclear translocation, but also alters the HSF DNA binding activity, thus delaying the heat shock response [10]. Interestingly, in HUVEC, H_2O_2 was also able to induce the NFκB p65 subunit nuclear translocation, but failed to induce NFκB DNA binding activity. Thus, the lack of NFκB activation by H_2O_2 was likely caused by oxidation of the p65/p50 on a sensitive thiol, and not by deficiency of the activation pathway. Cysteine-62 on p50 has been shown to be critical for DNA binding. Alternatively, p65 could be a target for oxidation as it has a cysteine in an analogous position to that in p50. In agreement with our observation, others have shown that pyrrolidine dithiocarbamate, a pro-oxidant which increased oxidized glutathione relative to reduced glutathione, did not interfere with the activation and nuclear translocation of NFκB, but rather oxidized NFκB and prevented DNA binding [11].

The effects of TNFα were also selective and were quite opposite to those exerted by H_2O_2. Thus, as expected, TNFα activated NFκB. TNFα also enhanced DNA binding activity of CRE binding proteins, but did not induce HSF activation. In addition, exposure to TNFα did not alter basal intracellular oxidant production in HUVEC, consistent with other reports [12,13]. These observations, together with the results obtained with H_2O_2, imply that, in our cells, the effects of TNFα was unrelated to any oxidant properties. They also suggest that a model for NFκB activation involving ROI is unlikely to be true for all cell types.

In accordance with previously published data [10,14,15], we observed that H_2O_2 and diamide led to a significant reduction in DNA binding activity of the three transcription factors, which was fully reversible with the reducing agent DTT. TRX, a thiol containing polypeptide of ~12 kDa which has a remarkable activity in thiol disulfide exchange reactions [16], in conjunction with NADPH and TR, was at micromolar concentration as active as DTT at millimolar concentration in providing reducing potential. However, both DTT and TRX failed to reactivate the CREP and NFκB after IAA treatment, consistent with alkylation being an irreversible process.

In conclusion, our results together with those reported by other workers suggest that HSF and NFκB may be under dual regulation. In the cytosol, a pro-oxidant signal would have a positive effect on HSF and NFκB activation and translocation to the nucleus. In the nucleus, these factors must be maintained in a reduced state for DNA binding to occur. The

equilibrium between oxidants and antioxidants within these compartments, which may be cell type specific, would explain the divergence in the literature regarding the effect of H_2O_2 on NFκB activation [17–21]. Our data also imply that the presence of H_2O_2 at sites of inflammation must be considered in analyzing the cytokine responses of vascular endothelium in inflamed tissues.

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