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# Tobacco Smoke Induces Coordinate Activation of HSF and Inhibition of NFκB in Human Monocytes: Effects on TNFα Release

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**Tobacco smoke (TS) exposure is a major risk factor for human disease, and macrophages of healthy smokers have a depressed capacity to release cytokines, including tumor necrosis factor (TNF)α. TS induces the synthesis of heat shock (HS)/stress proteins (HSP), and, in particular, of Hsp70. We determined whether Hsp70 induction by TS was mediated by the activation of the HS transcription factor, HSF. HSF activation has been shown to inhibit NFκB. Thus, we also determined the effects of TS on NFκB. U937 cells and human peripheral blood monocytes were exposed to TS, binding activities of the respective transcription factors were analyzed, and Hsp70 expression and TNFα release were determined in parallel. TS activated HSF, which was associated with Hsp70 overexpression and inhibition of NFκB binding activity and TNFα release. The altered cytokine profile observed in smokers may relate to an HSF/Hsp70-mediated inhibition of NFκB activity.** 

Tobacco smoke (TS) is the major single factor responsible for chronic lung diseases including cancer. Smoking impairs the immune response in the lung, and the pulmonary microenvironment of healthy smokers is often altered. The toxicity of TS is due to a variety of compounds among which nicotine, cadmium and reactive oxygen species (ROS). TS exposure represents a stressful condition, and we reported that it indeed leads, in mammalian cells, to the synthesis of the so-called heat shock (HS)/stress proteins (HSP), and in particular, of the cytosolic, inducible, 72 kDa HSP, Hsp70 (1, 2).

In human cells, HSP expression is regulated at both the transcriptional and the post-transcriptional level, and the type of regulation depends upon the stressor (3, 4, 5, 6, 7). The HS transcription factor(s) (HSF), an ubiquitous factor whose activation is independent of protein synthesis, is present in unstressed cells, in a non-DNA-binding, cytosolic form. Activation leads to the oligomerization, nuclear translocation and binding of HSF to the HS consensus element, HSE (5′-nGAAnnTTCnGAAn-3′) (8). Both HS and ROS induce HSP expression via the transcriptional activation of HSF, but the type of regulation activated by other selected stresses remains a matter of controversy (9, 5, 7).

HSP, and Hsp70 in particular, protect cells and tissues, both *in vitro* and *in vivo,* from the deleterious effects of numerous mediators of inflammation including ROS and TNFα (10, 11). While monocytes-macrophages are essential producers of both ROS and cytokines, macrophages of smokers, as well as macrophages exposed to TS *in vitro,* display reduced cytotoxicity and a depressed capacity for lipopolysaccharide (LPS)-induced cytokine release, including interleukin (IL)-1, IL-6 and tumour necrosis factor α (TNFα) (12, 13, 14). In normal cells, the release of these cytokines occurs subsequently to a cascade of signaling steps, the activation of the nuclear transcription factor NFκB being central to these events. NFκB was initially described as an activity that bound DNA fragments containing the decameric DNA sequence motif 5′-GGGACTTTCC-3′ (reviewed in 15). As HSF, NFκB normally exists in an inactive cytosolic complex, specifically bound to inhibitory proteins of the IκB family. Stimulation triggers the release of NFκB from IκB resulting in NFκB translocation to the nucleus where it activates the transcription of a number of genes encoding monocyte-macrophage stress response factors such as TNFα, other cytokines, c-*fos,* growth factors, the inducible · NO synthase, and other enzymes and factors involved in the control of the redox state of the activated cell, as well as the HIV long terminal repeat (16, 17).

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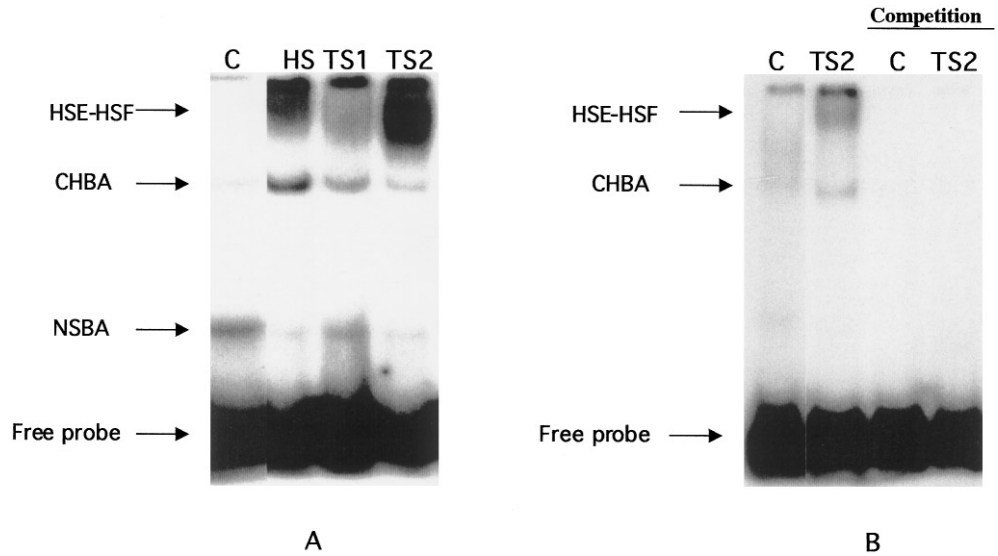


**FIG. 1.** Effects of TS on HSF binding activity in U937 cells and in human monocytes. (A) U937 cells were exposed to HS (44°C, 30 min) or to TS (0,24 puff/ml) for 1h (TS1) or 2h (TS2). Nuclear proteins (5$\mu$g) were subjected to gel mobility shift assay using $^{32}$P-HSE. Both HS and TS induced HSF-HSE binding activity. We observed constitutive HSF-HSE binding activity (CHBA) and non-specific binding activity (NSBA). This experiment was performed 4 times with similar results. (B) Human monocytes were exposed to TS (0,24puff/ml) for 2h (TS2). Nuclear proteins (5$\mu$g) were subjected to gel mobility shift assay using $^{32}$P-HSE. Human monocytes exposed to TS for 2h showed increased HSF-HSE binding activity and CHBA. The specificity of HSF-HSE binding activity was controlled by competition experiments with 100-fold molar excess cold HSE. This experiment was performed twice with similar results.

As for HSF, NF$\kappa$B is activated by proinflammatory and prooxidant factors (18, 19). However, *HSF activation* has been associated with *NF$\kappa$B inhibition*, suggesting a link between the regulatory pathways of the two transcription factors (20, 21, 22, 23). Two hypotheses have been proposed to explain this inhibition of NF$\kappa$B, either a direct inhibition of I$\kappa$B phosphorylation, or physical interactions between Hsp70 and NF$\kappa$B (21, 22, 24).

Here we investigated whether the induction of HSP synthesis by TS was mediated by HSF activation, and in such case, whether HSF activation by TS was associated with an inhibition of NF$\kappa$B binding activity and of TNF$\alpha$ release.

## MATERIALS AND METHODS

*Reagents.* [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol), Hyperfilm and the DNA 5′end-labeling kit were obtained from Amersham (Buckinghamshire, UK). Poly (dI-dC) and deoxyribonuclease-free bovine serum albumin were obtained from Pharmacia Biotech (Piscataway, NJ, USA). Protease inhibitors were purchased from Sigma Biochemicals (St. Louis, MO).

*Cells and culture conditions.* The human premonocytic line U937 was maintained in stationary cultures in RPMI 1640 medium (Gibco BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS; Gibco BRL), 2mM glutamine (Gibco BRL), 25 mM HEPES (Gibco BRL), 25$\mu$g/ml ampicillin (Gibco BRL) and 120$\mu$g/ml penicillin (Gibco BRL). Human peripheral blood mononuclear cells from healthy donors (buffy coats) were isolated by gradient centrifugation, monocytes purified by adherence (1) and maintained in RPMI-1640 medium, containing 10% fetal calf serum, 2mM glutamine and 25mM HEPES ($2.5\times10^6$ cells/ml). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 in air. For HS, cells were incubated in a water-bath at 44°C for 30 min then immediately prepared for nuclear extracts, or allowed to recover for 3h at 37°C for flow cytometry.

*Exposure to TS.* A peristaltic pump-smoke machine (Heinr. Borgwaldt RM1/G, Hamburg, Germany) was used to generate TS-bubbled phosphate buffered saline (PBS), from mainstream smoke of standard research cigarettes (reference 2R1, University of Kentucky, USA) through a puffing mechanism related to the human smoking pattern: one puff/minute (1 puff = 35 ml), each puff of 2 seconds duration. In the experiments presented here, the aqueous smoke fractions of one cigarette corresponded to 10 puffs (350 ml) bubbled into 5 ml of PBS. The final dilutions are expressed as puff/ml of culture medium. After extensive dose-response experiments (data not shown), we used in the experiments presented here, from 0.12 to 0.48 puff/ml. U937 cells or monocytes ($5\times10^6$) were exposed to TS-bubbled PBS for 1 or 2 h, then recovered by pipetting, respectively by gentle scraping with a rubber policeman for monocytes, and washed twice in PBS before nuclear extract preparation.

*Preparation of nuclear extracts and gel-mobility shift assays.* Preparation of nuclear protein extracts was as described (5, 6). Briefly, cells were collected, kept on ice for 10 min in a hypotonic lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM Phenylmethylsulfonylfluoride (PMSF), 10mM $\beta$-glycerophosphate and 1 $\mu$g/ml of antipapain, leupeptin and pepstatin. Nuclear pellets were resuspended in an extraction buffer (10 mM HEPES pH7.9, 400 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 mM $\beta$-glycerophosphate, 5% glycerol) with constant mixing for 30 min at 4°C. The samples were centrifuged at 10 000$\times g$ for 15 min at 4°C and the supernatant dialysed against a buffer containing 20 mM HEPES (pH7.9), 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 20% glycerol.

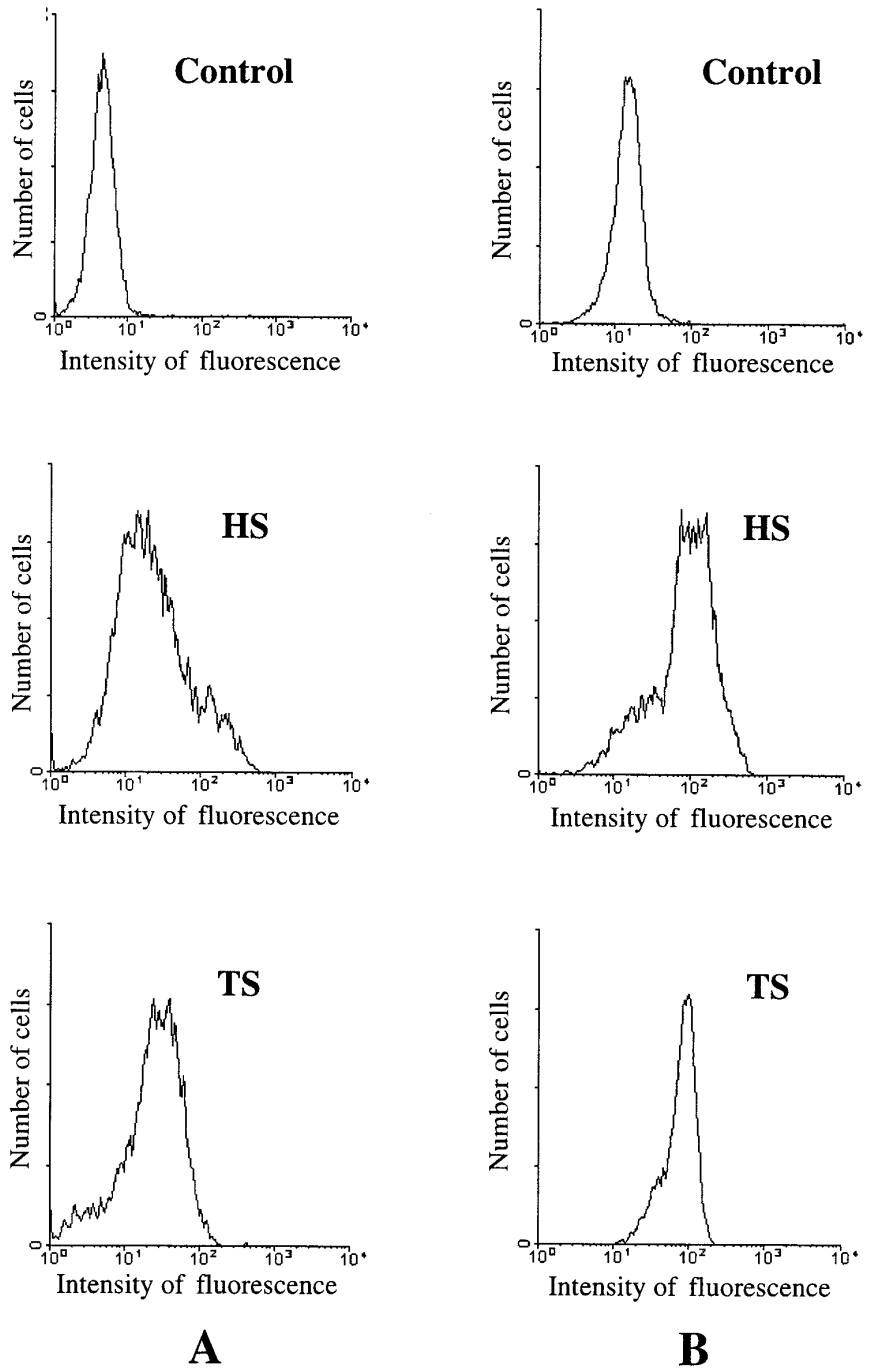


**FIG. 2.** TS-induced activation of HSF is followed by Hsp70 expression in U937 cells and in human monocytes. (A) U937 cells were exposed to HS (44°C, 30 min) or to TS (0,24 puff/ml, 3h), before being labeled with anti-human Hsp70. Analysis of immunofluorescence, detected with rabbit anti-mouse/FITC, was performed by flow cytometry. Percent cells expressing Hsp70 was 3,1 for control, 67,3 for HS and 73,9 for TS while mean fluorescence was respectively 29, 38,9 and 38,4. (B) Human monocytes were exposed to HS (44°C, 30 min) or to TS (0,24 puff/ml, 3h), before being labeled with anti-human Hsp70. Analysis of immunofluorescence was performed as for U937 cells. Percent cells expressing Hsp70 was 30,2 for control, 92,5 for HS, and 98,9 for TS while mean fluorescence was respectively 14,3, 118, and 76.

Samples were stored at −80°C. Nuclear extracts were then analyzed for HSE and NFκB binding activities by gel mobility shift assays. Binding reactions were performed for 30 min at 25°C by adding 5 μg of nuclear proteins to a mixture containing $10^6$ cpm of [$\gamma^{32}$P ATP] end-labeled, double-stranded HSE oligonucleotide (5′-GCCTCG-AATGTTCGCGAAGTT-3′) or $10^6$ cpm of [$\gamma^{32}$P ATP] end-labeled, double-stranded NFκB consensus sequence (5′-AGTTGAGGG-GACTTTCCCAGGC-3′) in 15 μl of dialysis buffer containing 2 μg of poly(d[I-C]) and 10 μg of bovine serum albumin. For the competition experiments, either a 100-fold molar excess of nonradioactive HSE or

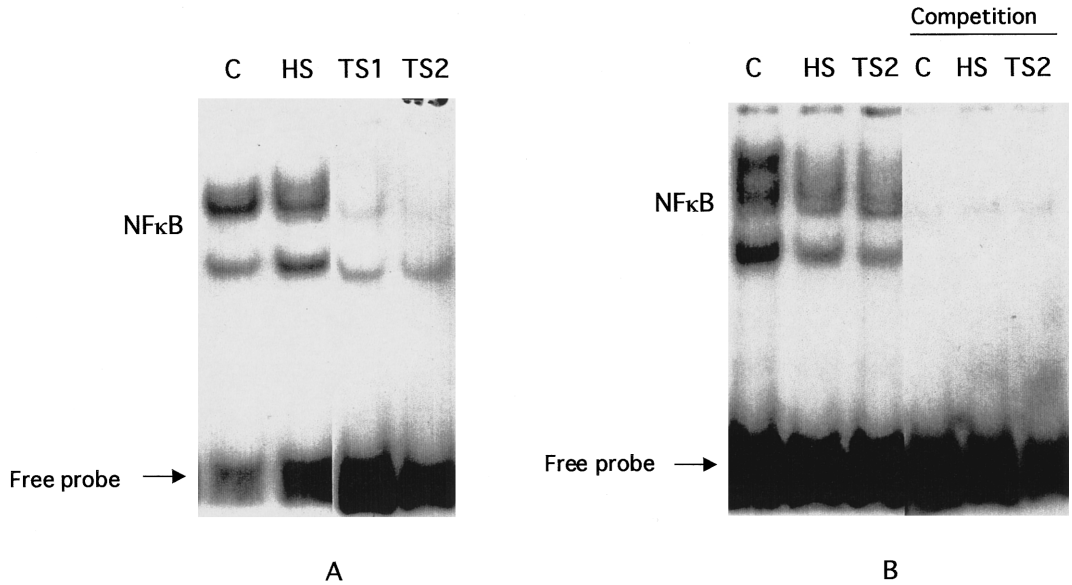


**FIG. 3.** Effect of TS on NFκB binding activity in U937 cells and in human monocytes. (A) U937 cells were exposed to HS (44°C, 30 min) or to TS (0,24 puff/ml) for 1h (TS1) or 2h (TS2), and nuclear proteins (5μg) were subjected to gel mobility shift assay. Basal NFκB binding activity was present in control U937 (two specific bands). HS, and even more so, TS exposure, for either 1 or 2h, inhibited NFκB binding activity. This experiment was performed 4 times with similar results. (B) Human monocytes were exposed to HS (44°C, 30 min) or to TS (0,24 puff/ml) for 2h (TS2), and nuclear proteins (5μg) were subjected to gel mobility shift assay. Basal NFκB binding activity was present in control monocytes (three specific bands). HS and TS similarly inhibited NFκB binding activity with the highest effect observed for the upper band. The specificity of NFκB-DNA binding activity was controlled by competition experiments with a 100-fold molar excess cold NFκB. This experiment was performed twice with similar results.

NFκB consensus sequence was used. Samples were electrophoresed on a nondenaturing 4% polyacrylamide gel at 160 volts for 2h, dried and autoradiographed.

*Detection of Hsp70 expression by flow cytometry.* Hsp70 expression was detected by flow cytometer as previously described (25, 2). U937 cells and monocytes were exposed to 0,24 puff/ml of TS for 3h, or to HS for 30 min, 3h recovery. Permeabilized cells were incubated with the anti-human Hsp70 antibody SPA810 from Stressgen (Victoria, Canada) at a dilution of 1:100 in PBS/BSA and stained with the rabbit anti-mouse/FITC (Dako, 1:30 dilution). Analysis was performed using a flow cytometer (Coulter, Miami, FL, USA) equipped with a single 488 nm argon laser. In all cases, a total of 5000 cells per sample were analyzed in listmode through 525 nm filter. Data were analyzed using the Elite software version 4.02.

*Determination of TNFα levels.* Human monocytes were exposed to TS-bubbled PBS at concentrations ranging from 0,12 to 0,48 puff/ml for 2 hours. Cells were then washed before adding LPS (10μg/ml) and incubating for 6 hours. TNFα release was measured in 100μl of human monocytes medium supernatant using the Pelkine Compact Human TNFα ELISA Kit (Sandwich-type enzyme immunoassay) (CLB, Amsterdam, The Netherlands), according to the manufactor's instructions.

*Cell viability.* Human monocytes were exposed to TS bubbled PBS at concentration of 0,24puff/ml for 2h. Cells were washed in PBS before adding LPS (10μg/ml) and incubating for 6h. Cells viability was then tested by measuring the release of lactate dehydrogenase in 100ml of cells medium supernatant using the LDH, MRP1 kit conforming to the recommendations of the manufactor (Boehringer, Mannhein, Germany).

*Statistical analysis.* Statistical comparisons between the groups were made using the two-tailed paired student's *t* test. A p value of less than 0,05 was considered to be significant.

## RESULTS

*Effects of TS on HSF binding activity.* In order to investigate whether HSP induction by TS was mediated by HSF activation, we examined HSF binding to its DNA consensus sequence in U937 cells and in human monocytes exposed to TS (0,24 puff/ml), using HS as positive control (Fig. 1). In U937 cells (Fig. 1A), control cell extracts contained no detectable HSE-binding activity (C). Extracts from heat shocked cells (44°C, 30 min) demonstrated HSF-HSE binding activity, which was also the case for TS exposure, whether for 1h (TS1) or 2h (TS2). As previously reported for ROS (6), HSF-HSE binding activity was consistently higher after 2h incubation with TS, as compared to 1h, indicating a delayed activation of HSF (6). For incubation times lower than 1h, no HSF-HSE binding was detected in these cells (not shown). Under the conditions of our experiments (HS and TS), we observed constitutive HSF-HSE binding activity (CHBA), as previously described (6). CHBA is prevented by competition with cold HSE (6). Non-specific binding activity (NSBA) was also detected in U937 cells; the lack of specificity of NSBA has been established in these cells by competition assays (6).

Similar experiments were performed on normal human monocytes. Cells were exposed to TS (0,24 puff/ml, 2h), nuclear proteins extracted, and HSF binding ac-

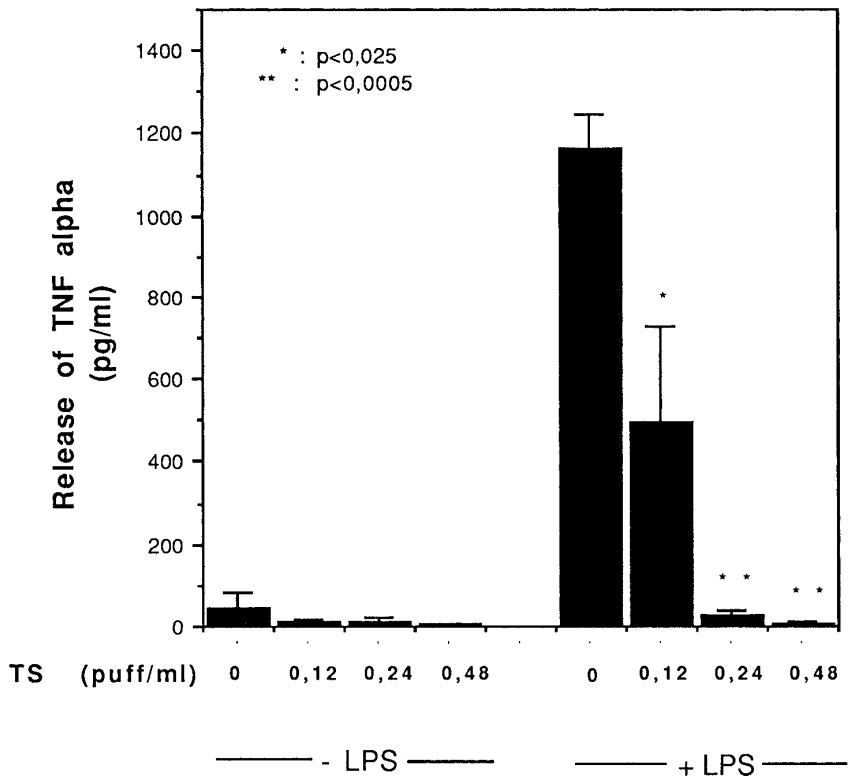


**FIG. 4.** TS inhibits basal and LPS-induced TNF$\alpha$ release in human monocytes. Monocytes were exposed to TS at concentrations ranging from 0,12 to 0,48 puff/ml for 2h, then the cells were washed and LPS (10 $\mu$g/ml) was added for 6h. TNF$\alpha$ was measured in the supernatants using a human TNF$\alpha$ ELISA kit. The height of the columns is proportional to the mean of the concentration (pg/ml/$10^6$ cells) of TNF$\alpha$ measured from 4 experiments ± SEM.

tivity determined as for U937 cells (Fig. 1B). Exposure of human monocytes to TS for 2h (TS2), but not for shorter incubation times, HSF-HSE binding activity was detected, as for HS. The specificity of the binding was demonstrated by competition with 100-fold excess of cold HSE. As for U937, CHBA was also present in human monocytes and was abolished by 100 fold molar excess of cold HSE. In contrast to U937, NSBA was not detectable in human monocytes.

*Effects of TS on Hsp70 expression.* To correlate HSF activation with HSP expression, cells were exposed to TS (0,24 puff/ml, 3h) or to HS (positive control) and Hsp70 expression analyzed by flow cytometry for both U937 and monocytes. Figure 2 represents the results of one representative experiment out of 15 and 10, respectively, for U937 cells (2A) and monocytes (2B). Basal levels of Hsp70 in control cells were different in U937 cells and in human monocytes: control U937 expressed very low levels of Hsp70 (3% positive cells) while 30% control monocytes expressed Hsp70. TS exposure induced Hsp70 in both types of cells. In U937 cells, HS induced Hsp70 in 67%, and TS, in 74% cells. In monocytes, HS induced Hsp70 in 93%, and TS, in 99% cells. The mean fluorescence (MF), corresponding to the amount of Hsp70 expressed per cell, was also cell-specific: in control U937 cells, MF was 29, raising to 39 after HS or TS exposure. In the control monocytes, MF was 14, raising to 118 after HS and 76 after TS exposure.

*Effects of TS on NF$\kappa$B binding activity.* HSF activation by hyperthermia, arsenite or prostaglandins has been previously shown to inhibit NF$\kappa$B (21). In order to study whether in the TS model of HSF activation, NF$\kappa$B was also inhibited in parallel to HSF activation, we determined the effect of TS exposure on the binding of NF$\kappa$B in unstimulated U937 cells and monocytes, using similar bandshift gel mobility assays as for HSF (Fig. 3). As previously described (26), basal NF$\kappa$B binding activity was present both in U937 cells (Fig. 3A), and in monocytes (Fig. 3B), though with different patterns (compare controls (C) in pannels A (U937 cells) and B (human monocytes)), probably corresponding to the various associations of NF$\kappa$B dimers (p65/p50) (21, 22, 27). Competition with 100-fold molar excess of cold NF$\kappa$B-consensus sequence abolished all the observed bands both in U937 (not shown) and in monocytes (Fig. 3B). In U937, NF$\kappa$B binding activity was inhibited by both HS and TS, the inhibitory effect being even more pronounced for TS than for HS. In human monocytes, HS and TS similarly inhibited NF$\kappa$B bind-

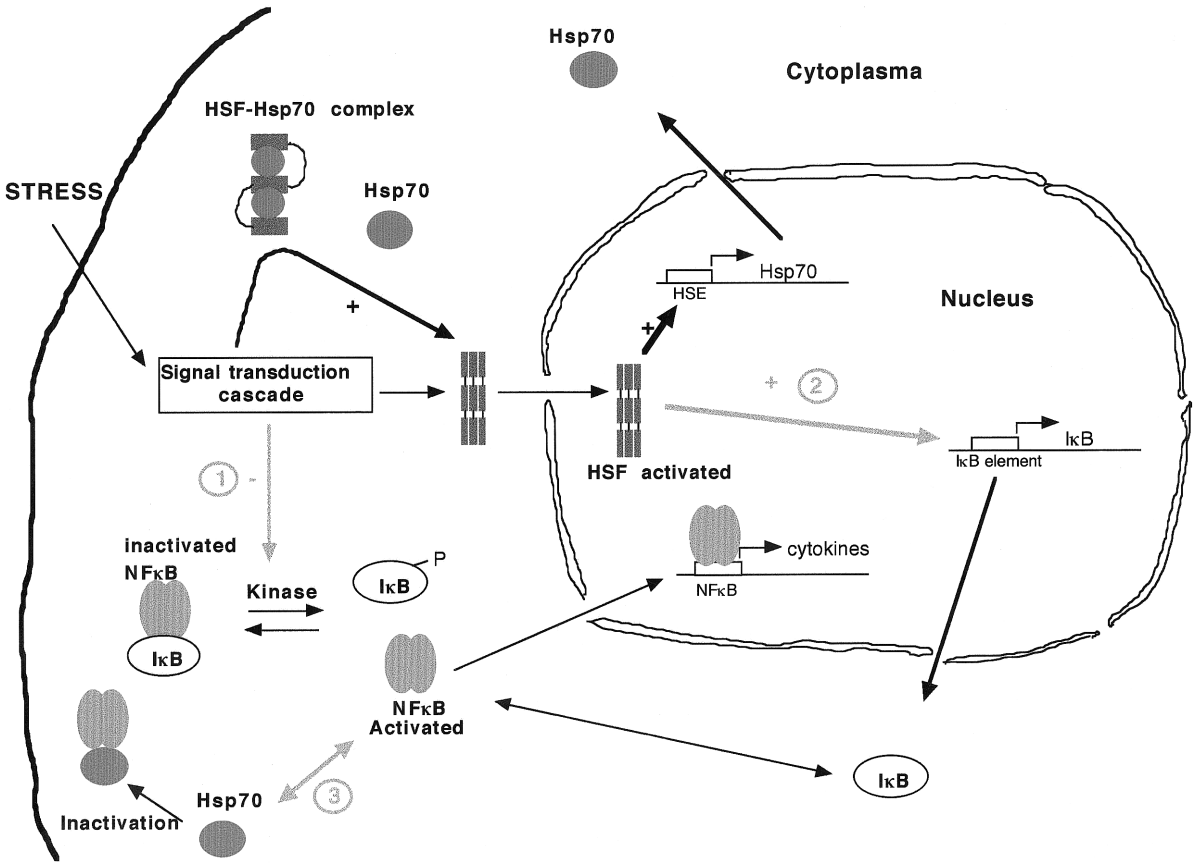


**FIG. 5.** Possible targets of NFκB inhibition along with HSF activation. Stresses as diverse as HS, cyclopentenone prostaglandins, arsenite, and TS, activate a signal transduction cascade leading to HSF activation, i.e., the release of HSF from its negatively regulated state, trimerization, and translocation to the nucleus. Inhibition of NFκB could result from at least 3 different mechanisms: (1) the stress response inhibits IκB phosphorylation via a second messenger of the stress signal transduction cascade; (2) HSF directly activates the transcription of *IκB*, leading to an increase in IκB expression; (3) Hsp70 chaperones the activated form of NFκB and partially substitutes IκB for NFκB inhibition.

ing, the inhibitory effect being most obvious for the upper band.

*Effects of TS on TNFα release by human monocytes.* On the one hand, NFκB is involved in the regulation of genes encoding cytokines, and on the other, altered cytokine profiles are observed in cigarette smokers. In order to determine whether *in vitro* exposure to TS altered cytokine production by human cells, we pre-exposed monocytes to TS (0,12 to 0,48 puff/ml, 2h). After washing, LPS (10μg/ml) was added for 6h, after which TNFα was quantified in the supernatants (Fig. 4.). In the absence of LPS, all concentrations of TS nearly abolished spontaneous TNFα release, although the differences were not statistically significant. Cells treated with LPS without TS pre-treatment released high levels of TNFα (1161 pmol/ml ± 87, mean ± SEM, n = 4). This release was significantly decreased by exposure to 0,12 puff/ml of TS and abolished at higher concentrations, while no sign of cell death, detected by the release of the lactate dehydrogenase of cells treated with TS and LPS compared to control, was observed under these experimental conditions (not shown). Inhibition of LPS-induced NFκB binding activity by TS was observed in parallel (not shown).

## DISCUSSION

Here we report that *in vitro* TS exposure activated HSF along with NFκB inhibition both in a pre-monocytic cell line and in normal human monocytes. Inhibition of NFκB by TS in human monocytes resulted in an inhibition of both spontaneous and LPS-induced TNFα release, which could relate to the altered cytokine profile observed in alveolar macrophages of smokers.

Inhibition of NFκB activation in parallel to HSF activation is specific neither for TS exposure nor for human monocytic cells. Other stresses, including HS, arsenite, and cyclopentenone prostaglandins inhibit NFκB in parallel to HSF activation in Hela, CEM or A549 cells (21, 22), suggesting that a common element of the pathway leading to HSF activation inhibits NFκB regardless of the inducing agent or the cell type.

Interestingly, despite the down-regulation of NFκB by HSF, the two transcription factors share similar regulatory characteristics such as the specificity and dual regulation by ROS, and the modulation of the binding activity by glutathione and thioredoxin (28, 6, 29).

TS contains a large variety of compounds which could activate HSF, among which ROS (5). However, ROS are unlikely to be involved in Hsp70 expression since the use of antioxidants such as quercetin or N-acetyl-L-cystein (NAC) prior to TS exposure, did not prevent the induction of Hsp70 (1). Interestingly, TS dramatically decreased intracellular glutathione levels, which could be restored by antioxidants, without any effect on Hsp70 expression (Polla, unpublished), further supporting our hypothesis that redox regulation is not involved in the effects of TS on the transcription factors considered. Cadmium is another compound of TS which could activate HSF via ROS (30). However, depletion of cadmium from TS using a chelating resin did not inhibit TS-induced Hsp70 expression, while cadmium-mediated induction was prevented (Vayssier, unpublished). While the specific compound(s) responsible for HSF activation by TS thus remain to be identified, this activation is likely to be ROS-independent.

The inhibition of NFκB coordinate to HSF activation could be targeted at different levels of the NFκB activation pathway and be mediated by specific signals of the stress response, by HSF itself, and/or by Hsp70. Specific signals activated during the stress response stabilize IκBα, inhibit its phosphorylation and prevent its degradation (21). HSF could contribute to an increase IκBα expression: indeed, the human IκBα promotor contains a contigous 20 bp segment that matches with HSE (21, 22). Alternatively, Hsp70 could chaperone NFκB, as a partial substitute for IκB (24). Along these lines, Feinsten et al (20) demonstrated that the transfection of glial cells with the human *hsp70* gene and the subsequent overexpression of Hsp70 leads to the suppression of the inducible NO synthase, which was directly associated with the inhibition of NFκB. The various possible mechanisms of NFκB inhibition by TS, along with HSF activation, are summarized in Fig. 5, and are the subject of further studies of this and other laboratories.

Inhibition of NFκB binding activity by TS correlated with a decrease of both basal and LPS-induced TNFα release by human monocytes. Downregulation of cytokine gene expression in mononuclear cells by the stress response, and of cytokine-mediated inducible NO synthase, have been shown before (31, 32, 33, 20). Hsp70 could also prevent TNFα release by intracellularly chaperoning the cytokine along its secretion pathway (34). Thus, TS could abolish TNFα release by a dual mechanism: on the one hand, by inhibiting NFκB and subsequent TNFα synthesis, and on the other hand, by inducing Hsp70, thus leading to intracellular TNFα retention. The impaired TNFα secretion secondary to TS exposure likely contributes to the increased incidence of infection and cancer in smokers. We propose that the decreased pulmonary defences of smokers relate to the coordinate induction of Hsp70 and inhibition of NFκB described here.

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