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# Effect of radical scavengers on TNFα-mediated activation of the uPA in cultured cells

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**Abstract.** Active oxygen, produced by cultured cells following stimulation with various growth factors, seems to be involved in signal transduction leading to cellular responses such as gene expression and growth modulation. In the present study, the intracellular oxidation state was measured in immortalized human endothelial cells (ECV304) after treatment with tumor necrosis factor (TNF)α, using a fluorescent dye and a laser-scanning confocal microscope. The intracellular oxidation state was increased 60 min after the addition of TNFα, and this increase was abolished by a radical scavenger, N-acetylcysteine (NAC), which is also a precursor of glutathione, and by pyrrolidine dithiocarbamate (PDTC). TNFα increased the steady state level of urokinase-type plasminogen activator (uPA), and NAC inhibited this increase at a dose that also inhibited the increase in the intracellular oxidation state. PDTC, on the other hand, did not affect the induction of the uPA gene by TNFα. These results suggest that intracellular glutathione level rather than the oxidation state is necessary for the induction of the uPA gene by TNFα.
**Key words.** Plasminogen activator; active oxygen; gene expression; radical scavengers; endothelial cells.

Cultured cells, other than phagocytic cells, produce active oxygen when stimulated with cytokines such as TNFα, IL-1[1,2], or TGFβ[3], or growth modifiers, including phorbol esters[4] and lipopolysaccharide[5]. Although reactive oxygen species (ROS) are generally toxic and damage cellular biomolecules, recent evidence indicates that ROS can act as bio-signals elicited by growth factors. Activation of the transcription factor NFκB by TNFα seems to be mediated in a ROS-dependent manner[6], and ROS produced from TNFα-treated cells inactivate a certain type of protein phosphatase, thereby increasing the phosphorylated form of the small molecular weight heat shock protein (HSP27)[7]. We have previously shown that hydrogen peroxide generated by TGFβ acted as one of the second messengers of phosphorylation in HSP27 (ref. 8) and in egr-1 gene induction[9]. Satriano et al. recently showed experiments using radical scavengers[10] suggesting that oxygen radicals act as second messengers for the expression of the JE gene in response to TNFα. Production of IL-8 by LPS in blood cells was also reported to be inhibited by dimethylsulfoxide, a scavenger of the OH radical[11].

We have been studying the biological roles of active oxygens in the expression of genes, especially early response genes[12,13], and we have found that the serum-response element (SRE, CArG box) seems to be responsible for the induction of c-fos by hydrogen peroxide[13]. However, the results above indicated that 'late response' genes are also regulated via ROS- mediated pathways. IL-1 and TNFα which increase ROS production, induce urokinase-type plasminogen activator (uPA)[14]. These cytokines are responsible for the acute inflammatory processes that often lead to fibrin deposition. The plasminogen activator cascade is a key component in fibrolysis, and neoplastic cells have been shown to express higher levels of proteolytic enzymes, giving them invasive properties[15]. The transcriptional regulatory regions of uPA have already been extensively analyzed, and PEA3 and AP-1 seem to be required for its transcription[16,17]. The activity of AP-1 is regulated by the redox state[18]. In the present study, we analyzed the effect of radical scavengers on the intracellular redox state and on the expression of the uPA gene in cells treated with TNFα.

## Materials and methods

**Cell culture and chemicals.** Spontaneously immortalized human endothelial cells, ECV304, were cultured in a $CO_2$-incubator in M199 medium supplemented with 10% fetal bovine serum on plastic dishes (Nunc, Denmark) as described by Takahashi et al.[19]. Cells were passaged by trypsinization. TNFα was obtained from Boehringer Mannheim GmbH (Germany); Cu/Zn SOD, catalase, NAC, and PDTC were purchased from Sigma Chemicals Co. (Saint Louis, Missouri, USA)

**Measurement of intracellular redox state.** Levels of the intracellular redox state were measured using a fluorescent dye, dichlorofluorescein diacetate (DCFH-DA), a non-polar compound that is converted into a nonfluorescent polar derivative (DCFH) by cellular esterases after being incorporated into the cells. DCFH trapped within the cells is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidases[20]. The fluorescence intensity of oxidized DCFH was employed as an index

of the levels of intracellular hydrogen peroxide. For assays, medium was replaced with Hanks solution containing 5 μM DCFH-DA at apropriate times after treatment of cells with TNFα. After 5 min incubation, the cells were visualized, and fluorscence intensity was measured with a BioRad MRC600 confocal laser scanning microscope (excitation at 513 nm, emmision at 535 nm). Relative fluorescence intensity was calculated using untreated control cells as standard.

**Extraction of RNA and analysis.** Total RNA was extracted by the guanidium/hot phenol method, electrophoresed, blotted and hybridized with nick-translated probes as described previously[21]. The probe used for the detection of human uPA mRNA was a subclone of the pHUK-1 gene [22] (1.0 kb Pst I/Bam HI fragment inserted into pUC18). Mouse α tubulin cDNA (Mα1)[23] was used to monitor the amounts of RNA.

## Results and discussion

**Effects of scavangers on intracellular oxidation state induced by TNFα.** Cells in the pre-confluent state were treated with 10 ng/ml of TNFα for 1 h in the absence or presence of scavengers, and the fluorescence intensity of DCFH was measured. Figure 1a and b shows that the intracellular oxidation state increased about three-fold, and that NAC at 40 mM inhibited the increase almost completely. Another scavenger, pyrolidine dithiocarbamate (PDTC), also inhibited the increase in the intracellular oxidation state at 0.1 mM (fig. 1c, d), whereas

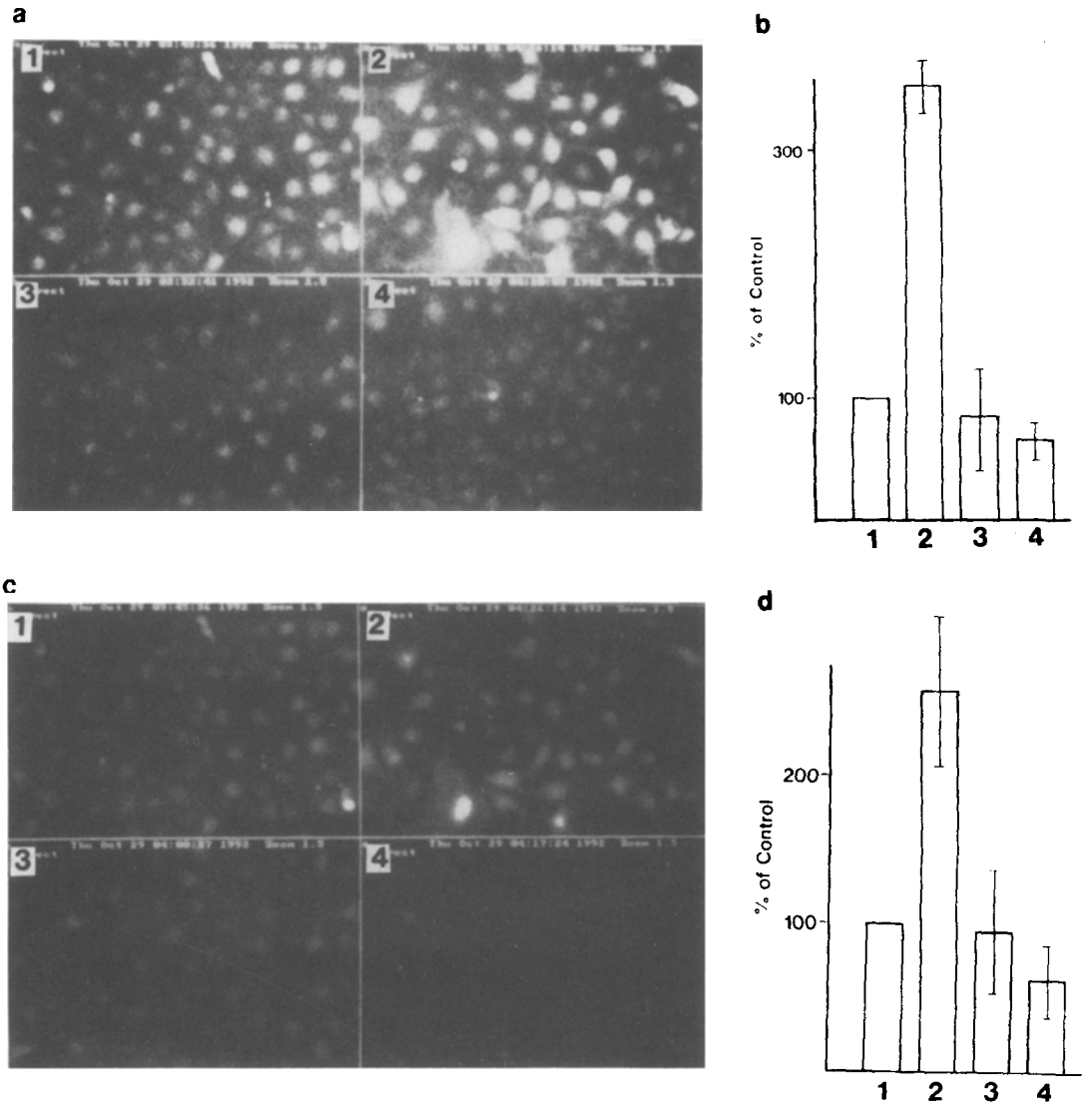


Figure 1. Change in redox state in ECV304 cells following treatment with TNFα, and effects of scavengers. Cells in the pre-confluent state were either untreated (*a* and *c*, panel 1), or treated with 10 ng/ml of TNFα in the absence (*a* and *c*, panel 2) or presence of scavengers for 1 h: *a* panel 3, TNFα + 40 mM NAC; *a* panel 4, 40 mM NAC; *c* panel 3, TNFα + 0.1 mM PDTC; *c* panel 4, 0.1 mM PDTC. Fluroescence intensity was visualized with a confocal microscope. The relative fluorescence intensity of each cell was measured, and the means of about 50 cells were calculated.
*b* and *d*: Results of calculation of *a* and *c*, respectively. The results show the means ± SD.

20 μg/ml SOD and 1200 U/ml catalase had essentially no effect (data not shown). In a previous study, we found that catalase was effective in inhibiting the production of hydrogen peroxide induced by tumor growth factor $\beta 1$[3,9]; catalase would not be expected to be incorporated into the cells, because of its high molecular weight. We speculated that hydrogen peroxide was secreted into the culture medium and that it acted from outside the cells[3]. The induction of IL-8 by LPs was inhibited by dimethyl-sulfoxide, a scavenger of the OH radical[10]. Thus, different extracellular signals may stimulate cells to produce different active oxygen species in different cellular compartments, and TNFα increases the cellular oxidized state by inducing the production of intracellular active oxygens.

**Effects of scavengers on the induction of uPA by TNFα.** We next examined the effects of the scavengers on the expression of the uPA gene induced by TNFα. NAC, which inhibited the increase in cellular oxidation state, also abolished the induction of uPA mRNA by TNFα (fig. 2). The time course of the change in uPA mRNA levels is shown in figure 3. When NAC was included in the culture medium, uPA mRNA was not induced at any time point examined up to 24 h following stimulation of cells with TNFα. NAC was effective in inhibiting uPA induction when added 1 h before or at the same time as TNFα, but was ineffective when added 1 h after the addition of TNFα (fig. 4) These results indicate that the inhibitory effect of NAC was confined to within 1 h after the cells were stimulated with TNFα. We then examined the effect of another scavenger, PDTC, on uPA-induction by TNFα; however surprisingly, PDTC did not affect uPA induction (fig. 5), although it effectively inhibited the increase in the intracellular oxidized state induced by TNFα (fig. 1c, d). PDTC is known to activate AP-1[24],

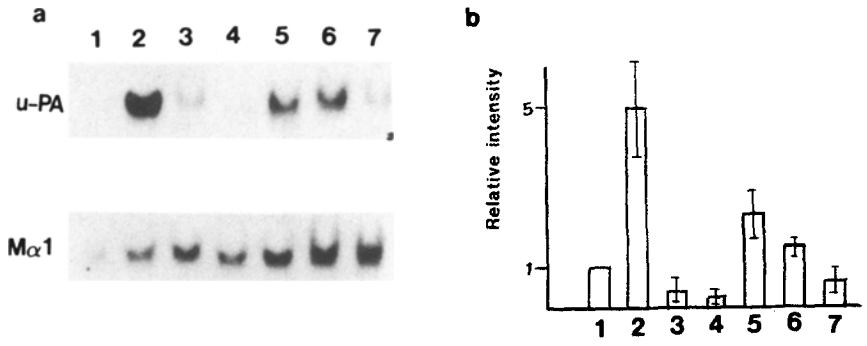


Figure 2. Effects of NAC on the induction of uPA by TNFα.
*a* Cells were either untreated or treated with 10 ng/ml TNFα in the absence or presence of NAC for 4 h. 1, untreated control; 2, TNFα; 3, 20 mM NAC; 4, 40 mM NAC; 5–7, TNFα + 5 mM, 20 mM or 40 mM NAC. Total RNA was extracted, and 20 μg/lane of RNA was electrophoresed, transferred to nylon filters, and hybridized with $^{32}$P-labeled uPA probe. Mα1 was used to monitor the amount of RNA in each lane. The filters were washed and autoradiographed.
*b* The autoradiograms were scanned, and the relative uPA mRNA levels were calculated on the basis of Mα1 mRNA levels. Vertical bars indicate the standard deviation obtained from three independent experiments.

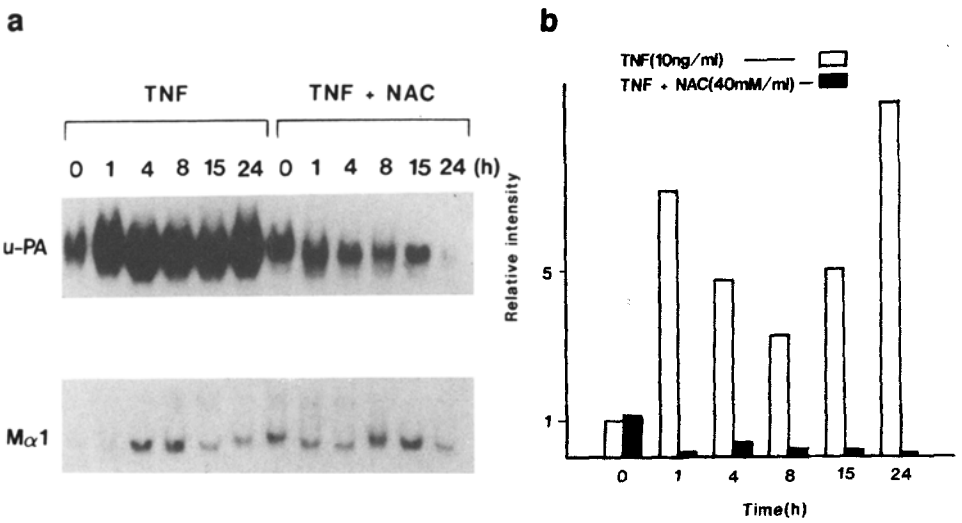


Figure 3. Time course of uPA induction. Cells were treated with 10 ng/ml TNFα for the indicated times, and the total RNA was analyzed as described in the legends to figure 2.

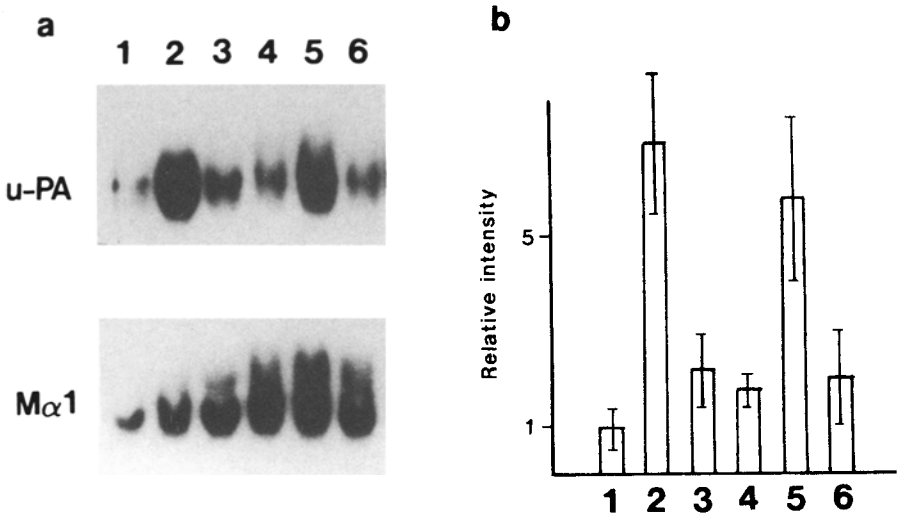


Figure 4. Effects of NAC added at different times following treatment with TNFα. Cells were either untreated (lane 1), or were treated with 10 ng/ml of TNFα (lane 2) or with 40 mM NAC (lane 6) for 4 h. NAC (40 mM) was added after 1 h before (lane 3), at the same time (lane 4), and 1 h after the addition of TNFα (lane 5). Total RNA was extracted and analyzed as described.

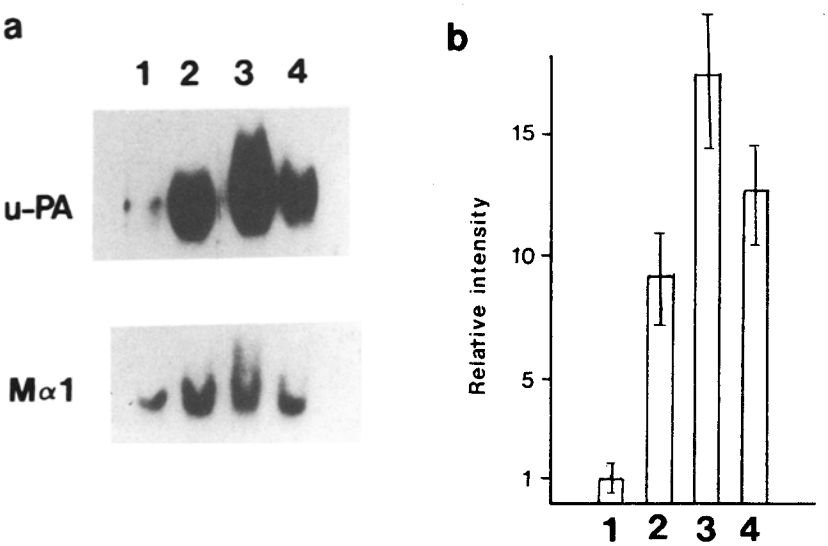


Figure 5. Effects of PDTC on the induction of uPA by TNFα. Cells were cultured in the absence (lane 1), or presence of 10 ng/ml TNFα without (lane 2) or with 0.1 mM PDTC (lane 3). Lane 4 shows PDTC alone.

and the increase in uPA mRNA level could be due to this effect.

NAC, a nontoxic drug, enters cells readily and serves both as a scavenger for reactive oxygen intermediates and as a precursor for glutathione[25]. Gilbert et al. proposed the gluthathione/disulfide exchange to be a second messenger of signal transductions[26]. The thiol-disulfide exchange provides a mechanism for transferring redox states between two thiol-disulfide pairs. Proteins or non-protein sulfhydryl groups may react with a disulfide to form a mixed protein disulfide. Glutathione is the most abundant thiol-disulfide redox buffer, and could participate in such an exchange. It thus seems possible that the increased levels of GSH caused by NAC modify cellular factors involved in uPA mRNA expression by TNFα.

Due to the limited number of cells, we were not successful in determining whether the effect of NAC was exerted at the transcriptional level. However, it has been shown that the transcriptional enhancer of the uPA gene contains PEA3 and AP-1 sites[16,17]. The transcription factors that bind to these elements could be possible candidates for the redox-based modification of uPA gene expression. Another possibility is that the stability of uPA mRNA was affected, since the stability of mRNA is also known to be redox-sensitive[27,28]. NAC could destabilize uPA mRNA, but we observed no effect of NAC on the rate of decay of uPA mRNA measured in the presence of actinomycin D (data not shown). The mechanism responsible for the effect of NAC is still unclear, and requires further examination. The expression of the uPA gene is reported to be

elevated in carcinomas[29], and the modulation of its expression by NAC could potentially prevent cancer metastasis.

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