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Short Communication

**REACTIVE OXYGEN SPECIES UPREGULATE EXPRESSION OF
PAI-1 IN ENDOTHELIAL CELLS**

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Abstract: Second messengers involved in the signal transduction pathway leading to induction of the plasminogen activator inhibitor (PAI-1) have not yet been well characterized. This study focuses on the mechanisms of regulation of PAI-1 expression by reactive oxygen species (ROS) in human endothelial cells. Inhibition of the tumor necrosis factor α (TNF α)-induced expression of PAI-1 by antioxidant N-acetyl-L-cysteine (NAC) indicated redox-sensitive mechanisms involved in the signalling pathway. Because TNF α induces PAI-1 production in endothelial cells, and NAC attenuated this response, we attempted to investigate the possible involvement of ROS in the activation of PAI-1 by TNF α . Upregulation of PAI-1 expression in endothelial cells by the stimulation with TNF α (50ng/ml) or H₂O₂ (10-200 μ M), observed by measurement of the antigen and mRNA levels, was reversed in the presence of NAC (20mM). The stimulatory effect of ROS was detected also at the level of the PAI-1 promoter in endothelial cells transfected with plasmid p800 LUC containing a PAI-1 promoter fragment (+71 to -800). The PAI-1 promoter activity was increased in the presence of ROS, and was suppressed by up to 75% in the presence of antioxidants [1]. On the basis of this study we can conclude that reactive oxygen species play an important role in a cytokine-induced activation of PAI-1 expression, and may act as a signal transduction messenger.

Key Words: Plasminogen Activator Inhibitor, Reactive Oxygen Species, Endothelial Cells

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INTRODUCTION

The initial response to vessel wall injury is characterized by the rapid deposition and activation of platelets, stimulation of the coagulation cascade as well as enhanced formation of reactive oxygen species, and precedes a more prolonged period of vascular repair [2]. Growth factors and cytokines released from activated platelets may contribute to thrombus formation and can also exert direct effects on the vessel wall and stimulate the formation of ROS [3,4]. Although ROS are cytotoxic in excess amounts, at low concentrations they serve as signalling molecules and second messengers affecting signal transduction and gene expression in a variety of processes including the response to injury [5-8]. Activation of the coagulation system leads to fibrin formation, which stabilizes the hemostatic plug, but is also required for wound healing and vessel wall repair. Plasminogen activator inhibitor-1 which regulates the fibrinolysis process, is markedly upregulated in endothelial cells by TNF α [9]. We showed that TNF α increased the PAI-1 mRNA and protein levels, and was also efficient in enhancing PAI-1 promoter activation in human endothelial cells. Inhibition of TNF α - induced expression of PAI-1 by antioxidants indicated redox-sensitive mechanisms involved in the signalling pathway. The exposure of endothelial cells to ROS caused enhanced PAI-1 expression and activity of the PAI-1 promoter. These findings demonstrate that signalling pathways stimulated by TNF α - induced PAI-1 expression are redox sensitive.

MATERIALS AND METHODS

Cell cultures

The human endothelial cell line EA.hy 926 was cultured in DMEM medium as previously described [10]. Cell viability was assessed by direct counting of trypan blue dye-excluding cells.

PAI-1 mRNA analysis

Total cellular RNAs were extracted from human endothelial cells using the Trizol reagent method, a single-step purification protocol [10,11]. The quality of the isolated total RNA was checked by 1% agarose - 2M formaldehyde gel electrophoresis. 1 μ g of total RNA was then used for cDNA synthesis by the SuperScript II RNase H Reverse Transcriptase System, employing oligo (dT) 12-18 primers. cDNA was amplified with forward and reverse primers for PAI-1 (5'GCTGAATTCCTGGAGCTCAG3', 5'CTGCGCCACCTGCTGAAACA3') or β -actin (5'GTGGGGCGCCCCAGGCACCA3' 5'CTCCTTAATGTACAGC-ACGATTTTC3') cDNA. The amplification products (35 cycles, each including 95°C, 30 s; 59°C, 30 s; 72°C, 30 s) were resolved on 6% polyacrylamide gel.

Cell treatment and cell lysates

Before treatment with any agents, the cells were starved for approximately 12 hours in DMEM medium supplemented with 0.1% fetal bovine serum.

Endothelial cells were stimulated with TNF α (50ng/ml) for 20 hours in the presence or absence of NAC (stock solution in PBS, pH 7.5). Preincubation of the cells with 10 or 20mM NAC was performed for 30 minutes. The PAI-1 antigen was determined by the ELISA test (Biopool).

Transient transfection of EA.hy 926 cells

Semiconfluent cell cultures in 6-well tissue culture plates were transfected with DNA constructs (plasmid p800LUC with the PAI-1 promoter obtained as a gift from Dr. D. J. Loskutoff, Scripps Clinic and Research Foundation, La Jolla, CA, USA) using the lipofectamine method as described before [12].

Data analysis

All values are expressed as mean \pm SE compared to controls and among separate experiments. Paired and unpaired Student's tests were employed to determine the significance of changes in the analysed parameters. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of reactive oxygen species on PAI-1 release from endothelial cells

TNF α strongly upregulates PAI-1 expression in the human endothelial cells line. To test whether TNF α -induced upregulation of PAI-1 involves production of reactive oxygen species, we attempted to modulate the effect of TNF α utilizing the inhibitor of ROS. In these experiments the cells were preincubated in the presence of antioxidant N-acetyl-L-cysteine (10mM or 20mM) for 30 minutes before treatment with TNF α (50ng/ml) for 20 hours. Activation of the cells with TNF α increased PAI-1 antigen release into the media (191.0 ± 9.7)% of control. Preincubation of the cells with 10mM NAC caused the inhibition of TNF α -induced PAI-1 accumulation by (47.6 ± 6.9)%, but 20mM NAC reduced the antigen level by (53.0 ± 4.1)% (Fig. 1A).

PAI-1 expression was increased when the cells were incubated with hydrogen peroxide. After treatment of the cells with 100 μ M or 200 μ M H₂O₂ for 30 minutes, PAI-1 expression was increased to (162.0 ± 4.1)% or (183.0 ± 8.4)% of control, respectively. Thus, hydrogen peroxide upregulates PAI-1 expression, and this effect is abolished in the presence of the cell permeant antioxidant NAC. NAC (20mM) inhibited the increase in PAI-1 accumulation induced by 100 μ M or 200 μ M H₂O₂ to (38.0 ± 3.4)% or (26.0 ± 2.9)%, respectively (Fig. 1B).

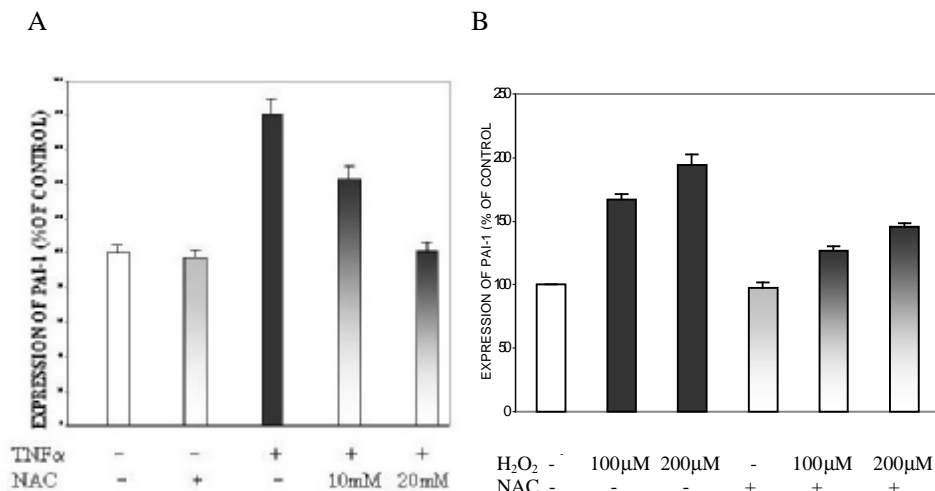


Fig. 1. Effect of antioxidant on PAI-1 release from endothelial cells after activation with TNF α and hydrogen peroxide. A) Endothelial cells were preincubated for 30 minutes with 10 or 20mM of N-acetyl-L-cysteine and stimulated with TNF α (50ng/ml) for 20 hours. B) Endothelial cells were exposed for 20 hours to increasing concentration of H₂O₂ (100 or 200 μ M) in the absence or presence of NAC (20mM). Experimental culture medium was assayed for PAI-1 concentration immediately or stored frozen at -70°C until use.

Effect of reactive oxygen species on synthesis of PAI-1 mRNA

Inhibitory effect of antioxidants on TNF α -induced expression of PAI-1 was also well documented by RT PCR analysis of PAI-1 mRNA. The cells were incubated for 4 hours with TNF α or H₂O₂ in the presence or absence of NAC.

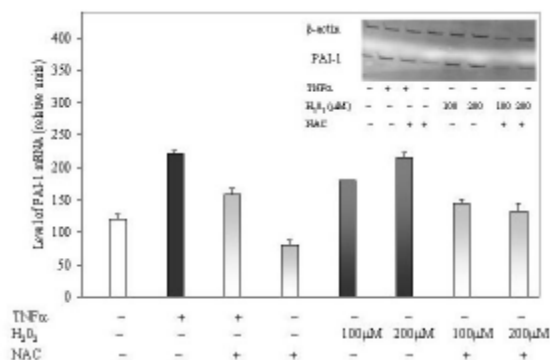


Fig. 2. Effect of TNF α and H₂O₂ on PAI-1 mRNA in human endothelial cells. Data represent the mean value \pm S.E.M. of six independent experiments. Representative pattern of rt-PCR analysis of actin and PAI-1 mRNA is shown as insert.

Changes in PAI-1 mRNA were estimated and compared to the β -actin mRNA levels. Treatment of the cells with TNF α or H₂O₂ significantly increased expression of PAI-1 mRNA, but did not affect the actin mRNA level. Antioxidant NAC significantly reduced the PAI-1 mRNA level in EA.hy 926 (Fig. 2).

Effect of reactive oxygen species on PAI-1 gene promoter activity

To study the responsiveness of the PAI-1 promoter to reactive oxygen species, endothelial cells were transfected with plasmid p800LUC containing a PAI-1 promoter fragment corresponding to positions from +71 to -800. In order to investigate whether ROS could have a functional effect on PAI-1 expression, a p800LUC construct containing the fragment of the PAI-1 promoter was transfected into an endothelial cell together with the internal control pSV. Functional studies with a firefly luciferase gene as a reporter were performed in unstimulated endothelial cells, and in cells stimulated for 12 hours with TNF α (Fig. 3A) or hydrogen peroxide (Fig. 3B). To determine whether reactive oxygen species were involved in the increased expression of PAI-1 induced by TNF α (50ng/ml) or hydrogen peroxide (100 μ M, 200 μ M), endothelial cells were preincubated with antioxidant NAC (10mM or 20mM) for 30 minutes. As shown in Fig. 3A, TNF α increased PAI-1 promoter activity by 14.5-fold as determined using the luciferase reporter gene. NAC (10mM or 20mM) caused reduction of TNF α induced PAI-1 promoter activity by 5.5- and 12.5-fold, respectively. PAI-1 is regulated by many factors *in vivo* and it is an acute phase reactant. TNF α is thought to be involved in mediating the PAI-1 acute phase response *in vivo* and may act *via* reactive oxygen species.

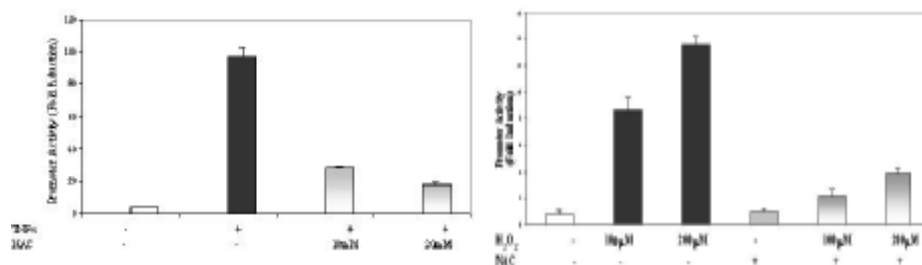


Fig. 3. Effect of TNF α (A) and hydrogen peroxide (B) on PAI-1 promoter activity

Results of this study suggest a possible mechanism through which TNF α induces PAI-1 synthesis in the endothelial cell line. TNF α increased the synthesis of PAI-1, but antioxidant NAC completely abolished this induction. To determine whether reactive oxygen species were directly involved in the increased expression of PAI-1 induced by TNF α , the cells were incubated with

hydrogen peroxide. TNF α can induce both superoxide and hydrogen peroxide production [13-15], and in the present study, the N-acetyl-L-cysteine, a membrane-permeant antioxidant inhibited TNF α -induced PAI-1 expression. Second messengers involved in signal transduction pathways leading to induction of PAI-1 have not yet been well characterized. We previously reported that the MAPK cascade was involved in the signal transduction pathway which modulates PAI-1 expression [16]. Because TNF α and reactive oxygen species had a similar effect on the expression of PAI-1, a common mechanism may be operating in the signal transduction pathways involved in induction of PAI-1 by TNF α in endothelial cells. Furthermore, data have been presented that PAI-1 protein expression is under the control of NF- κ B [17], and the NF- κ B sequence competes with the *ins/del* sequence of the PAI-1 promoter for binding of nuclear proteins [18]. Thus, one might speculate that the putative element in the PAI-1 promoter may be also responsive to ROS. The results suggest that ROS are involved in a pathway leading to activation of PAI-1 expression by TNF α .

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