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Original Research Communication

Anethole Dithiolethione Regulates Oxidant-Induced Tyrosine Kinase Activation in Endothelial Cells

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ABSTRACT

Interaction between neutrophils and endothelial cells is one of the first steps in the functional response of polymorphonuclear neutrophils (PMN), and is necessary for their migration toward damaged tissues. PMN activation, leading to their adhesion to and migration between endothelial cells, is part of a complex phenomenon that can be altered in pathological situations such as the ischemia-reperfusion syndrome, in which large numbers of PMN are recruited to the tissue and release reactive oxygen species (ROS) near the vessel wall. ROS have been implicated in the pathogenesis of various inflammatory diseases. The increased adhesion of PMN to ROS-stimulated endothelial cells involves an increase in tyrosine phosphorylation of a tyrosine kinase focal adhesion kinase ($p125^{FAK}$) and several cytoskeleton proteins, including paxillin and p130 cas. We examined the role of glutathione (GSH) in the regulation of this adhesion pheonomenon and in the increased tyrosine phosphorylation induced by ROS. For this purpose we used anethole dithiolthione (ADT), which increases the glutathione synthesis by activating γ-glutamyl-cysteine synthetase. We found that ADT reduced both PMN adhesion to ROS-stimulated human umbilical vein endothelial cells (HUVEC) and tyrosine phosphorylation of $p125^{FAK}$ and paxillin. ADT increased redox status by increasing intracellular GSH content in oxidized cells. These results show that GSH can reverse the effect of oxidation on tyrosine kinase activation and phosphorylation, and thus plays an important role in cell signaling. They also confirm the antioxidant activity of ADT. Antiox. Redox Signal. 2, 789–799.

INTRODUCTION

THE VASCULAR ENDOTHELIUM forms a barrier between tissues and blood and is constantly subjected to a variety of mechanical and chemical stresses. In some pathological situations, such as acute respiratory distress syndrome and ischemia-reperfusion injury, reactive oxygen species (ROS) are inappropriately released by inflammatory cells close to endothelial cells. Following ROS stimulation, expression of membrane adhesion proteins on endothelial cells is modified (Sellak *et al.*, 1994). The endothelium loses its selective barrier functions, leading to microvessel dysfunction and inflammatory cell infiltration. During ischemia-reperfusion, organs such as the heart and lungs are invaded by large numbers of neutrophils, with excessive release of ROS that partly accounts for cellular injury (Granger *et al.*, 1989; Suzuki *et al.*, 1989).

Neutrophil migration into tissues requires their adhesion to the vascular endothelium. This process is controlled by adhesion molecules located both on neutrophils and on endothelial cells; they are expressed following cell

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stimulation by various inflammatory molecules such as cytokines, endotoxin, thrombin, growth factors, and ROS (Carlos and Harlan, 1994). These events induce structural and morphologic changes, such as actin fiber reorganization and cytoskeleton modifications (Springer, 1990; Diamond *et al.*, 1990; Otey *et al.*, 1990; Carpen *et al.*, 1992; Ridley and Hall, 1992; Rankin and Rozengurt, 1994) that occur concomitantly with a change in the oxidized glutathione/reduced glutathione (GSSG/GSH) ratio in the relevant cells (Kirlin *et al.*, 1999).

In adherent cells, cytoskeleton organization involves a tyrosine kinase, focal adhesion kinase ($p125^{FAK}$), activated by integrin engagement. $p125^{FAK}$ is a 125-kDa protein tyrosine kinase that is crucial for transmission of signals from the focal adhesion to the cytoplasm after cell attachment (Kornberg *et al.*, 1992; Schaller *et al.*, 1992). After activation, $p125^{FAK}$ interacts with a variety of focal adhesion macromolecules, including the adaptor proteins, paxillin and p130cas, which are phosphorylated on a tyrosine residue by the complex focal adhesion kinase $p125^{FAK}$/p60src (Schaller *et al.*, 1992; Schaller and Parsons, 1995), leading to the formation of adhesion complexes. $p125^{FAK}$ also interacts with the cytoskeleton proteins α-actinin, vinculin, tensin, and talin, and with signal transduction molecules such as Src, Fyn, and PI 3-kinase (Turner *et al.*, 1990; Sakai *et al.*, 1994; Turner and Miller, 1994; Polte and Hanks, 1995; Salgia *et al.*, 1995; Schaller and Parsons, 1995; Vuori and Ruoslahti, 1995; Harte *et al.*, 1996).

Previously, we have shown that endothelial cells submitted to oxidative stress increase their adhesiveness for resting neutrophils (Sellak *et al.*, 1994; Franzini *et al.*, 1996). Indeed, endothelial cells are able to respond to ROS stimulation by increasing the activity of several tyrosine kinases and phosphatases, but it is not known how they are activated. It is not known either whether ROS act directly on the activity of a tyrosine kinase or if the redox status of the cells plays an important role.

Anethole dithiolethione (ADT) is a synthetic sulfide compound with antioxidant properties. It can act as an anticarcinogenic drug (Wattenberg, 1985; Egner *et al.*, 1994), an inhibitor of lipid peroxidation (Mansuy *et al.*, 1986; Christen *et al.*, 1995), and a protective agent against radiation- and ROS-induced cytotoxicity (Warnet *et al.*, 1989; Bouthillier *et al.*, 1996; Drukarch *et al.*, 1997; Khanna *et al.*, 1998). ADT is an activator of the enzyme γ-glutamyl-cysteine synthetase, which is involved in GSH synthesis (Ansher *et al.*, 1983; Warnet *et al.*, 1989), and of glutathione-*S*-transferase (Kensler and Groopman, 1996). ADT is thus able to maintain the redox status of ROS-stimulated cells by increasing the intracellular level of GSH.

The aim of this study was to determine the mechanism of tyrosine phosphorylation of $p125^{FAK}$ and paxillin induced by oxidative stress, using ADT as a regulator of intracellular GSH content.

MATERIALS AND METHODS

Chemicals

Hanks' balanced salt solution, with or without Ca^{2+} and Mg^{2+} (HBSS or HBSS wo) was from Gibco (Paisley, UK). Bacto-gelatin was from DIFCO (Detroit, MI). Six-well and 24-well gelatin-coated plates were from Falcon (Polylabo, Strasbourg, France). Anti-focal adhesion kinase ($p125^{FAK}$) and anti-paxillin (PAX) monoclonal antibodies were from Transduction Laboratories (Interchim, Montlucon, France). Monoclonal phosphotyrosine antibody [anti-Tyr(*P*)] and rabbit antimouse-immunoglobulin G (IgG) protein A agarose beads were from UBI-Biovalley (Lake Placid, NY). The ECL western blotting detection system, rainbow markers high range, and sheep antimouse horseradish peroxidase monoclonal antibody were from Amersham Life Sciences (Courtaboeuf, France). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Metaphosphoric acid was from Fisher Scientific Co (Springfield, NJ). All other products were from Sigma (St Louis, MO). ADT was from Solvay-Parma laboratory (Suresnes, France). Calcein-acetoxymethyl (calcein-AM) and 6-carboxy 2,7-dichlorodihydrofluorescein diacetate were from Molecular Probes (Eugene, OR).

Cell preparation

Endothelial cells were prepared as previously described (Sellak *et al.*, 1994). Briefly, human umbilical cord veins were treated with

0.05% collagenase to harvest endothelial cells (HUVEC). HUVEC were grown in M199 medium supplemented with fetal calf serum (FCS) (20%), L-glutamine (2 m*M*), Fungizone (5 μg/ml), penicillin (50 IU/ml), and streptomycin (50 μg/ml) at 37°C with 5% CO_2 in air. The medium (M199 with 10% human serum) was changed every second day until confluence. HUVEC were identified by phase-contrast light microscopy showing the typical cobblestone monolayer of cells and their ability to release von Willebrand factor. Cells were harvested after trypsin-EDTA treatment (0.05% and 0.02%, respectively) and seeded in 24- or 6-well gelatin-coated plates. Only monolayers of secondary cultures that were tightly confluent were used. Polymorphonuclear neutrophils (PMN) were isolated and purified from healthy human venous blood as previously described (Sellak *et al.*, 1994).

Cell viability and oxidation

Viability was measured with a calcein assay as previously described (Noël-Hudson *et al.*, 1997) with slight modifications. Briefly, HUVEC were stimulated in 24-well plates with Hx-XO for the times indicated, and the supernatant was then washed out. Five hundred microliters of 2.5 μM calcein-AM in Hanks' solution was added to each well for 30 min. Fluorescence was quantified on a Fluostar plate reader (BMG, LabTechnologies, France) using wavelengths of 480 nm for excitation and 520 nm for emission. Values were expressed as mFU units per well.

Intracellular oxidation was as 6-carboxy, 2,7-dichlorodihydrofluorescein diacetate (carbo-DCFH-DA) fluorescence. HUVEC were loaded with 10 μM carbo-DCFH-DA before ROS stimulation for 30 min. Fluorescence was determined on the plate reader, at wavelengths of 480 nm for excitation and 520 nm for emission (Haugland, 1996).

PMN adhesion to HUVEC

PMN adhesion to HUVEC was measured as previously described (Sellak *et al.*, 1994). Briefly, PMN (10^6/ml) were added to 24-well plates containing washed HUVEC that had or had not been incubated for 24 hr with ADT (5 to 100 μM) and exposed for 30 min to Hx-XO (Hx, 2×10^{-4} *M*, and XO, 4.5 mU/ml). After 2 min of centrifugation (1,800 rpm at 4°C) and 15 min of contact, unbound PMN were removed by four washes with HBSS and recovered by centrifugation. Pellets were lysed with 1% Triton X100 and sonicated (3×10 sec). Adherent PMN were collected with a rubber policeman, lysed, and sonicated. Myeloperoxidase (MPO) in adherent and non-adherent PMN fractions was measured as described elsewhere, with slight modifications. Briefly, MPO was determined as the increase in optical density at 460 nm resulting from the reaction of hydrogen peroxide (H_2O_2) (400 μM) with orthodianisidine (160 μM) in citrate buffer (100 μM, pH 5.5). A standard curve for PMN MPO was established to correlate optical density with the number of adherent PMN; the detection limit was 1,000 PMN/ml.

GSH measurement

GSH was measured in 24-well plates as described in (Browne and Armstrong, 1998), with modifications. Briefly, after treatments, supernatants were discarded and 300 μl of an aqueous solution containing 1.67 g/100 ml of metaphosphoric acid (Fisher Scientific Co, Springfield, NJ), 200 mg/100 ml of EDTA (Sigma), and 30 g/100 ml of NaCl was added to the cells. The plates were kept at −20°C until assay. Cells were scraped free with a rubber policeman and centrifuged.

Ten microliters were used in the assay in 96-well plates in 200 μl of GSH buffer (0.1 *M* NaH_2PO_4, 5 m*M* EDTA, pH 8) and 10 μl of *o*-phthalaldehyde reagent (1 mg/ml methanol). After 15 min at room temperature, the plate was read on the fluorometer plate reader at λex: 350 nm and λem: 420 nm. The values shown were calculated from a standard curve of GSH (0–50 mg/100 ml).

Tyrosine phosphorylation measurement

HUVEC in six-well 0.2% gelatin-coated plates were washed twice with HBSS and incubated with Hx-XO (Hx, 2×10^{-4} *M*, and XO, 4.5 mU/ml for 30 min or 2.5 mU/ml for 60–120 min), in HBSS for various times, in the presence of 100 μM sodium orthovanadate solu-

tion. Hypoxanthine oxidation by xanthine oxidase (4.5 mU/ml), generated around 3 nmol/ml per min of $^{\cdot}O_2^-$ and 50 nmol/ml of H_2O_2 over a 30-min period. Details of control treatments and experiments involving other drugs are given in the Results section. After stimulation, HUVEC viability was examined by means of calcein incorporation, as indicated above. No cytotoxic effect or cell detachment was observed. HBSS was then removed and cells were lysed in 50 μl of lysis buffer (10 m*M* Tris/HCl, pH 7.6, 50 m*M* NaF, 30 m*M* sodium pyrophosphate, 1% Triton X-100, 1 m*M* PMSF, 100 m*M* orthovanadate, 5 m*M* EDTA, and 50 m*M* NaCl). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with antiphosphotyrosine antibody. When indicated, HUVEC were preincubated with various ADT concentrations (20, 50, and 100 μM) for 24 hr prior to stimulation.

Immunoprecipitation

Confluent cultures of HUVEC in six-well gelatin-coated plates were washed twice with HBSS wo, then incubated or not incubated with 100 μM ADT for 24 hr before treatment with Hx-XO (Hx, 2×10^{-4} *M*, and XO, 4.5 mU/ml) in HBSS for the times indicated, and lysed at 4°C in 50 μl of cold immunoprecipitation buffer containing 10 m*M* Tris/HCl, pH 7.6, 50 m*M* NaF, 30 m*M* sodium pyrophosphate, 1% Triton X-100, 1 m*M* PMSF, 100 μM orthovanadate, 5 m*M* EDTA, and 50 m*M* NaCl for 30 min. Lysates were sonicated and centrifuged at $15{,}000 \times g$ for 10 min to remove debris, and the protein concentration was determined with the BioRad assay; the supernatants were incubated for 2 hr at 4°C with a monoclonal antibody (mAb) directed against Tyr(P), $p125^{FAK}$ or PAX (0.5–2 $\mu g/1.5 \times 10^6$ cells for each antibody) as indicated, while shaking gently on a wheel. The immune complexes were collected on rabbit antimouse IgG/Protein A agarose beads and incubated overnight at 4°C under agitation. Immunoprecipitates were then washed three times with immunoprecipitation buffer, collected in 80 μl of electrophoresis sample buffer (63 m*M* Tris-HCl, pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 8.7% glycerol) and boiled for 5 min before electrophoresis.

Western blotting

For SDS-PAGE, 20–50 μl of each sample (20 μg protein) and molecular weight standards were subjected to electrophoresis on 9% polyacrylamide gel by the method of Laemmli (1970). Following electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were incubated in blocking solution, Tris-buffered saline (TBS) (10 m*M* Tris, 100 m*M* NaCl, pH 7.5) containing 5% nonfat dried milk and 0.1% Tween-20 for 1 hr at room temperature, and then incubated with this blocking solution containing 250 ng/ml anti-$p125^{FAK}$ or 25 ng/ml anti-PAX mAbs overnight at 4°C. When anti-Tyr(P) mAb (1:4,000 dilution, 25 ng/ml) was used, the blocking solution contained 5% bovine serum albumin (BSA) instead of milk. The blot was washed three times with TBS containing 0.1% Tween-20, and incubated with a sheep anti-mouse secondary antibody coupled to horseradish peroxidase at 1:10,000 dilution in blocking solution for 1 hr at room temperature, followed by three additional washes with 0.1% Tween-20 in TBS. Detection was done on the Amersham ECL detection system and Hyperfilm MP (Amersham Life Science, Courtaboeuf, France). Tyrosine phosphorylation was quantified using NIH Image software.

Statistical analysis

All experiments were performed in duplicate or triplicate and repeated three times. Results are given as means ± standard deviation. Data were compared using Student's *t*-test and analysis of variance (ANOVA).

RESULTS

ADT effect on PMN adhesion to hypoxanthine/XO-stimulated endothelial cells

HUVEC simulated with Hx-XO (Hx, 2×10^{-4} *M*, and XO, 4.5 mU/ml) for 30 min were preincubated for 24 hr with various concentrations of ADT (5–100 μM), and PMN adhesion to these cells was measured. Figure 1 shows that a 10% adhesion rate was obtained with PMN layered on unstimulated HUVEC, compared to 23% after 30 min of Hx-XO stimula-

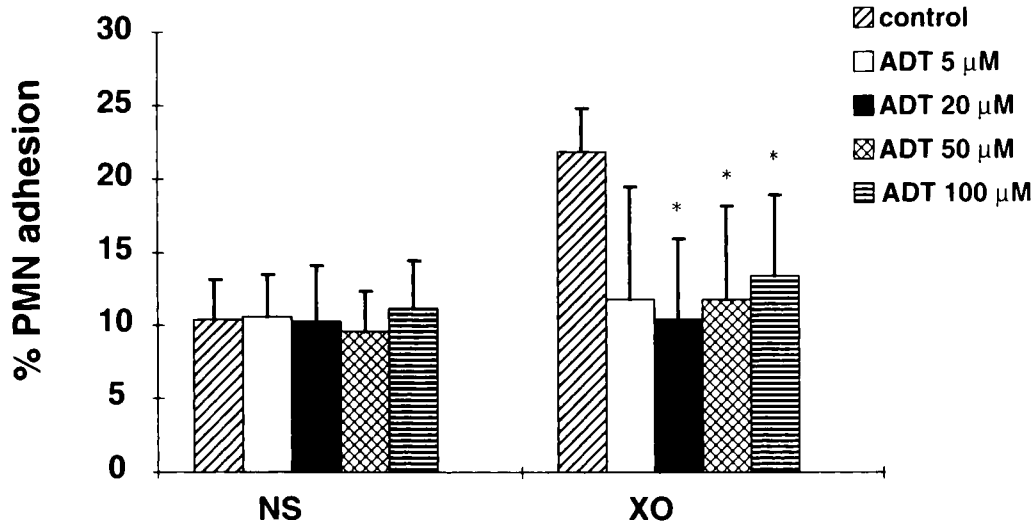

FIG. 1. Adhesion of neutrophils to Hx-XO-treated endothelial cells (HUVEC). Endothelial cells were stimulated with Hx-XO (2×10^{-4} *M*, 4.5 mU ml^{-1}) in 24-well gelatin-coated plates for 30 min in serum-free HBSS, pH 7.4, at 37°C or with HBSS buffer alone, and with or without ADT (5–100 μM) pretreatment for 24 hr, as indicated. Neutrophils were added (10^6/ml per well) to the wells and left for 15 min at 37°C. Adhesion was determined by myeloperoxidase measurement. Values are the means ± SD of at least three determinations ($n = 3$). *$p < 0.001$ versus Hx-XO-stimulated HUVEC.

tion, which was inhibited by 5, 20, 50, and 100 μM ADT. HUVEC were then preincubated with 100 μM ADT for 24 hr and stimulated for various times (60–120 min) with Hx-XO. ADT (100 μM) also inhibited PMN adhesion to HUVEC when the latter were stimulated for 60 and 90 min (by 33 and 44%, respectively; results not shown).

Cell viability and oxidation by Hx-XO: effect of ADT

Intracellular oxidation and cell viability were measured as a function of the XO concentration (2.25, 4.5, and 9 mU/ml) during a 30-min incubation period. Cell viability was slightly, but not significantly, decreased. The increased fluorescence of carbo-DCFH was XO concentration-dependent and statistically significant (XO 4.5 mU/ml and 9 mU/ml versus control, $p < 0.01$) (Fig. 2).

When the cells were pretreated with 20, 50, and 100 μM ADT for 24 hr, oxidation decreased independently of the ADT concentration and returned to the control value (Fig. 3).

GSH content of ROS-stimulated HUVEC: effect of ADT

HUVEC were stimulated with various XO concentrations (2.25, 4.5, and 9 mU/ml) for 30 min, and GSH was then measured. The amount of GSH fell as oxidation increased (Fig. 4). The cells were then pretreated for 24 hr with 20, 50, and 100 μM ADT and stimulated for 30 min with Hx-XO 4.5 mU/ml. The amount of GSH fell in cells stimulated with Hx-XO, and strongly increased in cells pretreated with ADT, in a concentration-dependent manner up to 50 μM (Fig. 5).

Viability of these cells, measured in terms of calcein incorporation, was unaffected by the different treatments (Figs. 4 and 5). GSH values are given as a ratio to cell viability. We controled that ADT added in the GSH samples did not modify the GSH measurements. The amount of GSH for nonstimulated cells was 67.5 $\mu M/10^6$ cells, and for ROS-treated cells (XO, 4.5 mU/ml) it was 24 $\mu M/10^6$ cells.

ADT effect on total tyrosine phosphorylation of HUVEC proteins

Tyrosine-phosphorylated proteins were measured in HUVEC preincubated with 20, 50, and 100 μM ADT for 24 hr and stimulated for 30 and 60 min with Hx (2×10^{-4} *M*) and XO (4.5 mU/ml). Figure 6A shows the increase in total tyrosine phosphorylated proteins (lane 3) in 30-min ROS-stimulated cells as compared to nonstimulated cells (lane 1) and to nonstimulated cells treated with 100 μM ADT (lane 2).

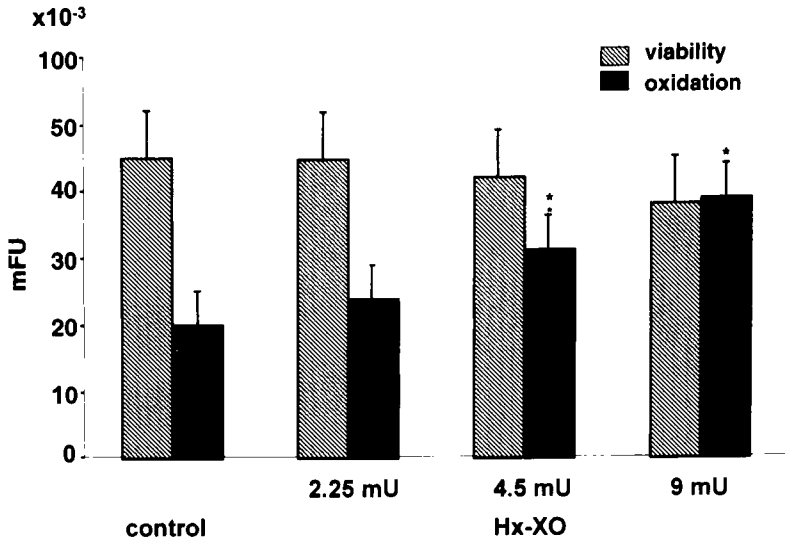

FIG. 2. HUVEC oxidation and viability. HUVEC were stimulated for 30 min in 24-well gelatin-coated plates with various Hx-XO concentrations (2.25, 4.5, and 9 mU/ml) in HBSS. Cell viability was measured as 30-min calcein (2.5 μM) incorporation as described in Materials and Methods and expressed in mFU/ml. Oxidation was measured by 10 μM carbo-DCFH fluorescence incubated for 30 min with cells before stimulation. Fluorescence was measured in a fluorescence plate reader (Fluostar) at λem = 480 nM and λex = 520 nm. Values are the means ± SD of three determinations (*$p < 0.01$ versus control cells).

When the cells were pretreated with ADT (20, 50, 100 μM) for 24 hr, protein tyrosine phosphorylation decreased in an ADT concentration-dependent manner (lanes 4, 5, and 6, respectively). The amount of protein loaded on the gel was the same for each sample (Fig. 6B).

ADT effect on tyrosine phosphorylation of p125^{FAK} and paxillin

We then investigated the phosphorylation of two proteins involved in focal adhesion: focal adhesion kinase and paxillin (a cytoskeleton protein). HUVEC were treated for 24 hr with 100 μM ADT and stimulated for 30 and 60 min with Hx-XO (XO 4.5 mU/ml; 3 nmol $^{\cdot}O_2^-$/min/ml); p125^{FAK} and paxillin were immunoprecipitated and blotted against an anti-phosphotyrosine antibody or anti-p125^{FAK} and anti-paxillin, respectively. Tyrosine phosphorylation of p125^{FAK} was increased (Fig. 7A) after Hx-XO treatment for 30 and 60 min, and decreased when the cells were pretreated with

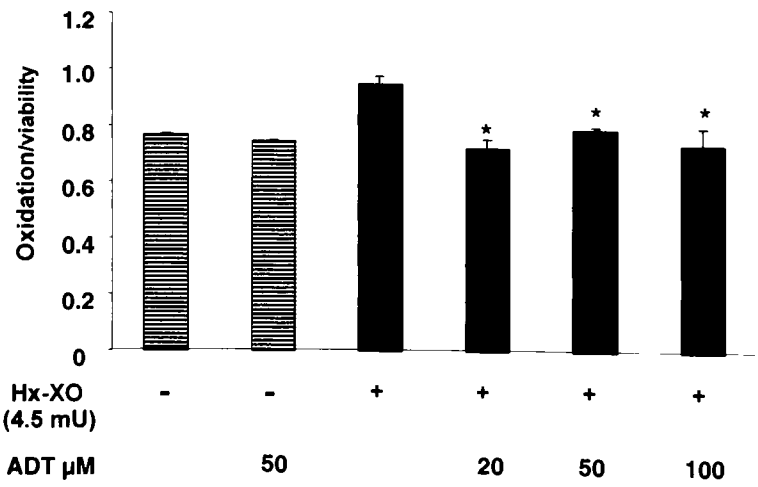

FIG. 3. Effect of ADT on HUVEC oxidation. HUVEC were incubated for 24 hr with various concentrations of ADT in M199 medium (20, 50, and 100 μM) before stimulation for 30 min with Hx-XO (4.5 mU/ml) in serum-free HBSS, pH 7.4, at 37°C. Intracellular oxidation and viability were measured as described in Fig. 2 (carbo-DCFH and calcein, respectively). Values are the means ± SD of three-determinations.

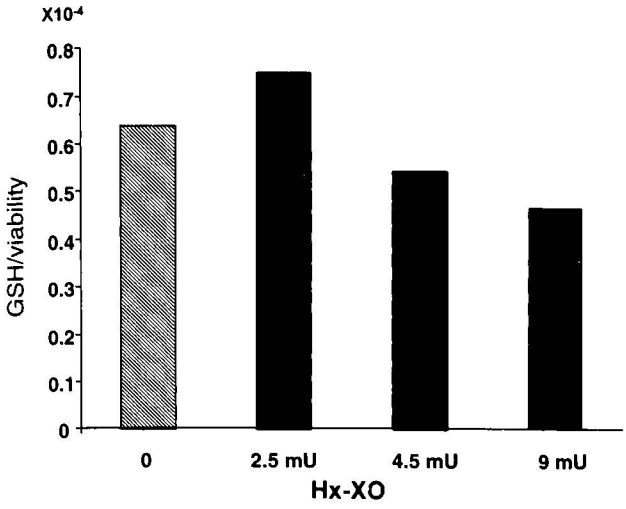

FIG. 4. **Reduced GSH in oxidized HUVEC.** HUVEC were stimulated with three different concentrations of XO (2.25, 4.5, and 9 mU/ml) for 30 min in serum-free HBSS, pH 7.4, at 37°C and GSH was measured in 24-well plates by using a fluorometric technique as described in Materials and Methods. Values are means ± SD of three determinations.

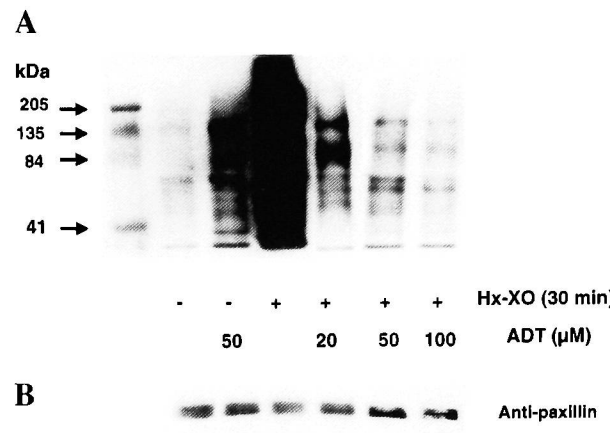

FIG. 6. **Total tyrosine phosphorylation of ROS-stimulated HUVEC.** (**A**) Endothelial cells were stimulated with Hx-XO (2×10^{-4} *M*, 4.5 mU ml^{-1}) in 24-well gelatin-coated plates for 30 min in serum-free HBSS, pH 7.4, at 37°C, with or without ADT (20, 50, and 100 μM), and incubated for 24 hr in M199 medium before stimulation. Total tyrosine phosphorylation of the HUVEC homogenate was blotted with an anti-phosphotyrosine antibody (25 ng/ml). The cells were not treated (lane 1), incubated with 50 μM ADT (lane 2), or stimulated with Hx-XO (2×10^{-4} *M*/4.5 mU per ml) for 30 min (lanes 3–6) and then incubated with ADT (20, 50, or 100 μM) for 24 hr (lanes 4, 5, 6). (**B**) The same volume of each total cell homogenate was loaded on a gel and the blot was probed with an anti-paxillin antibody (25 ng/ml).

100 μM ADT. The amount of $p125^{FAK}$ protein loaded was the same in each lane (Fig. 7B). Tyrosine phosphorylation of paxillin was also increased after ROS stimulation for 30 and 60 min, but this was only slightly inhibited by 100 μM ADT (Fig. 8A). The same amount of protein was loaded in each lane (Fig. 8B).

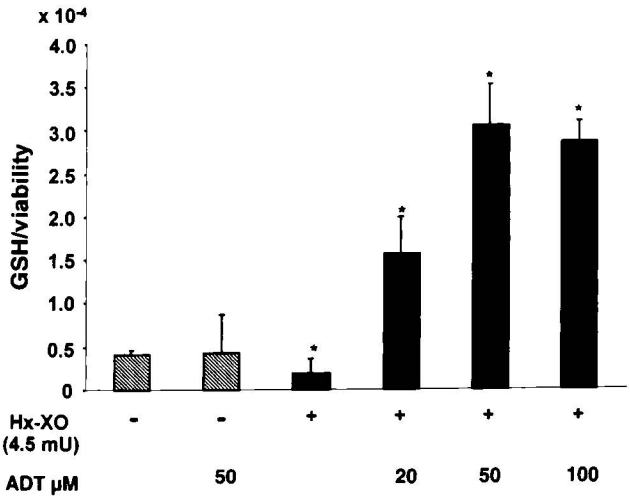

FIG. 5. **Effect of ADT on reduced GSH.** HUVEC were preincubated for 24 hr with 20, 50, or 100 μM ADT in M199 medium before stimulation with Hx-XO 4.5 mU/ml for 30 min in serum-free HBSS, pH 7.4, at 37°C, and GSH was measured as described in Fig. 4. Values are means ± SD of three determinations.

DISCUSSION

In this study, we found that ADT inhibited the ROS-induced increase in PMN adhesion to endothelial cells, together with the increased intracellular oxidation, the decreased intracellular GSH level, and the increase in protein tyrosine phosphorylation of $p125^{FAK}$ and paxillin. ADT can effectively be considered as an antioxidant, because it increases the amount of intracellular GSH by its activation of the enzyme γ-glutamyl-cysteine synthetase (Ansher *et al.*, 1983; Warnet *et al.*, 1989).

$P125^{FAK}$ is a member of a non-receptor protein tyrosine kinase family that plays a role in regulating changes in actin cytoskeleton organization. $P125^{FAK}$ in adherent cells is associated with focal adhesion; phosphorylation of its tyrosine and that of the cytoskeleton-associated protein paxillin and p130cas can be activated by β1 and β3 integrins, and by a variety of regulatory peptides and lipids mediating cell growth and differentiation (Schaller *et al.*, 1992; Guan and Shalloway, 1992; Hanks *et al.*, 1992). Its activation by various stimuli leads to acti-

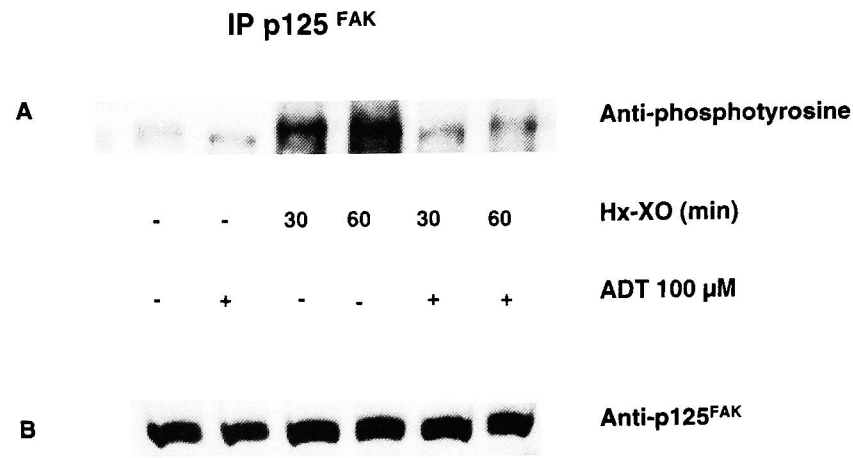

FIG. 7. ADT effect on ROS-induced tyrosine phosphorylation of p125^{FAK}. (**A**) Endothelial cells were stimulated with Hx-XO (2×10^{-4} *M*, 4.5 mU ml^{-1}) in 24-well gelatin-coated plates for 30 min and 60 min in serum-free HBSS, pH 7.4, at 37°C, with or without preincubation with 100 μM ADT in M199 medium. After treatment, they were immunoprecipitated with an anti-p125^{FAK} antibody (1 μg/1.5 $\times$ 10^5 cells). The blot was probed with an anti-phosphotyrosine antibody (25 ng/ml). (**B**) The same volume of each immunoprecipitate was loaded on a gel and the blot was probed with an anti-p125^{FAK} antibody (25 ng/ml).

vation of another tyrosine kinase, pp60src, which seems to be responsible for tyrosine phosphorylation of paxillin and p130cas (Schaller and Parsons, 1995; Schlaepfer and Hunter, 1998). Previously, we have shown that this focal adhesion complex is activated by an increase in intracellular oxidation (Gozin *et al.*, 1998), which induces a fall in intracellular GSH and leads to an increase in the GSSG/GSH ratio (Kokura *et al.*, 1999). GSSG plays an important role in intracellular signaling; in particular, it acts directly on the activity of two tyrosine kinases, p56^{lck} and p59^{fyn} (Dröge *et al.*, 1994). There is considerable evidence that agents altering the GSH concentration affect transcription of detoxification enzymes, as well as cell proliferation and apoptosis (Kirlin *et al.*, 1999). The increase in tyrosine phosphorylation of p125^{FAK}, paxillin, and p130cas in HUVEC after cell treatment with Hx-XO for 15 min is immediately inhibited when cells are treated with antioxidants (Gozin *et al.*, 1998). We found here

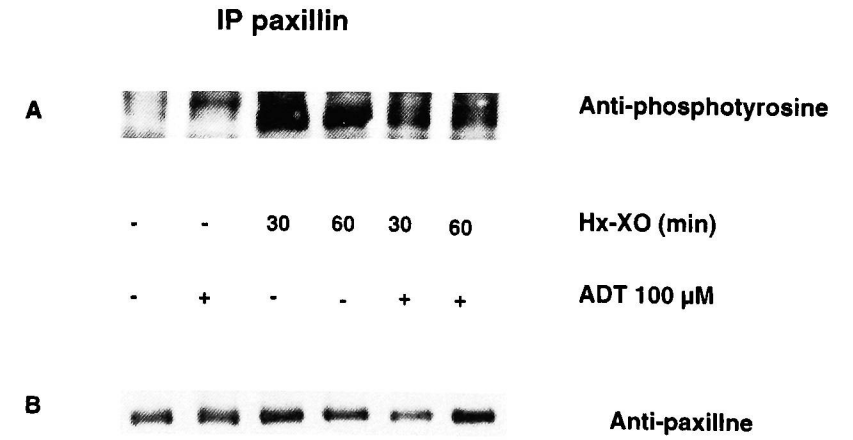

FIG. 8. ADT effect on ROS-induced tyrosine phosphorylation of paxillin. (**A**) Endothelial cells were stimulated with Hx-XO (2×10^{-4} *M*, 2.5 mU ml^{-1}) in 24-well gelatin-coated plates for 30 and 60 min in serum-free HBSS, pH 7.4, at 37°C, with or without preincubation with 100 μM ADT in M199 medium. After treatment, they were immunoprecipitated with an anti-paxillin antibody (1 μg/1.5 $\times$ 10^5 cells). The blots were probed with an anti-phosphotyrosine antibody (25 ng/ml). (**B**) The same volume of each immunoprecipitate was loaded on a gel and the blot was probed with an anti-paxillin antibody (25 ng/ml).

that ADT inhibition of tyrosine phosphorylation of these proteins took place after 30 min of oxidation. ADT is thought to decrease the GSSG/GSH ratio by acting on the enzyme γ-glutamyl-cysteine synthetase (Ansher *et al.*, 1983; Warnet *et al.*, 1989).

When HUVEC were oxidized by xanthine oxidase, we found a net increase in oxidation and a parallel decrease in GSH. ADT reversed this phenomenon, showing that ADT effectively acts on GSH synthesis. With the increase in oxidation and the decrease in the GSH level, we also obtained an increase in tyrosine phosphorylation of cytoskeleton proteins ($p125^{FAK}$ and paxillin), which was also reversed by ADT. Finally, PMN adhesion to ROS-stimulated endothelial cells, which was increased by oxidation, was decreased by ADT, indicating that this adhesion could be dependent on both the intracellular GSH level and tyrosine phosphorylation of cytoskeleton proteins.

Thus, the decrease induced by ADT in intracellular oxidation-induced tyrosine phosphorylation and in PMN–endothelial cell adhesion point to a role of the GSSG/GSH ratio in this intracellular signaling induced by oxidative stress. GSSG increases as a consequence of reduction in GSH in the presence of oxidants; Recently, it has been shown that the intracellular GSH level and the GSSG/GSH ratio play an important role in the induction of the neutrophil–endothelial cell adhesion response, involving synthesis of adhesion molecules on HUVEC (Kokura *et al.*, 1999). In this latter study, the authors showed that an increase in reduced GSH down-regulated the rapid phase (15 min) of adhesion, whereas late-phase adhesion (6 hr) correlated positively with the GSSG/GSH ratio. These two phases are regulated by the intracellular GSH level, and changes in the latter cause transcription-independent and transcription-dependent surface expression of adhesion molecules on endothelial cells. We found that ADT had an effect on PMN adhesion to endothelial cells after both 15 min and 60 min of stimulation, and at the same time had an inhibitory effect on tyrosine phosphorylation of cytoskeletal proteins after 30 min of stimulation. This could be explained by an inhibitory action of GSH on the activation of $p125^{FAK}$ activity, which would in turn inhibit the expression of adhesion molecules (*e.g.*, P-selectin) on endothelial cells.

Tyrosine phosphorylation of $p125^{FAK}$ was markedly inhibited by ADT, whereas that of paxillin was only slightly inhibited. pp60src is a tyrosine kinase, whose activity is related to the activity of $p125^{FAK}$ but can remain active even if $p125^{FAK}$ becomes inactive (Schaller and Parsons, 1995; Schlaepfer and Hunter, 1998). This could explain why, when $p125^{FAK}$ is inhibited, paxillin is still phosphorylated. GSH might thus be able to inhibit the activity and phosphorylation of $p125^{FAK}$ and, as a consequence, also inhibit part of the phosphorylation of cytoskeleton proteins.

Understanding the effect of ADT on ROS-induced PMN adhesion to HUVEC and tyrosine phosphorylation of cytoskeleton proteins will help to identify the pathway involved in these processes. Because oxidative stress induces a decrease in intracellular GSH (which is involved in redox status), GSH is a good candidate signal transducer. It remains to be determined if activation of PMN–endothelium adhesion correlates with an increase in tyrosine phosphorylation of $p125^{FAK}$.

ABBREVIATIONS

ADT, Anethole dithiolethione; anti-Tyr(P), anti-phosphotyrosine antibody; BSA, bovine serum albumin; GSH, GSSG, reduced gluta thione, oxidized glutathione; HBSS, Hanks' balanced salt solution; HUVEC, human umbilical vein endothelial cell; Hx, hypoxanthine; mAb, antibody; MPO, myeloperoxidase; PMN, human polymorphonuclear neutrophils; PMSF, phenylmethylsulfonylfluoride; $p125^{FAK}$, focal adhesion kinase; PAX, paxillin; p130cas, p130 Crk-associated substrate; ROS, reactive oxygen species; TBS, Tris buffered saline; Xo, xanthine oxidase.

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