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Cell adhesion and integrin expression are modulated by oxidative stress in EA.hy 926 cells

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Abstract

The effects of oxidative stress on integrin-mediated cell adhesion to the extracellular matrix (ECM) and related apoptosis were investigated using the EA.hy926 endothelial cells treated (or not) with two oxidants: the hypoxanthine/xanthine oxidase system (HX/XO) or the *tert*-butyl hydroperoxide (*t*-BHP) which both increased cell apoptosis. Cell adhesion onto vitronectin (Vn) and fibronectin (Fn) was increased at low concentrations of HX/XO (up to 5 mU/ml) or *t*-BHP (up to 125 μM) and prevented ROS-induced apoptosis. Flow cytometry analysis of integrin expression showed that the expression of integrin αv and α5 subunits was, respectively, increased and decreased. Cell adhesion inhibition experiments using function-blocking monoclonal antibodies against integrin subunits indicated that αvβ1 and αvβ3 integrins were involved in adhesion of cells to Vn, and αvβ3 integrin played a major role in oxidant-treated cells. For adhesion to Fn, α5β1 and αvβ1 integrins were required for oxidant-treated cells. Taken together, the results suggest that reactive oxygen species (ROS) produced either by HX/XO or *t*-BHP could affect expression and/or activation of specific integrins in the interaction of EA.hy926 cells with ECM.

Keywords: *Reactive oxygen species, apoptosis, endothelial cell adhesion, extracellular matrix, integrins*

Introduction

Integrins are heterodimers consisting of noncovalently associated transmembrane α and β subunits [1,2]. In cell–extracellular matrix (ECM) interactions, integrins are known to be implicated in the control of cell adhesion and cell survival [3–7]. Integrins binding to ECM proteins is dependent on signal transduction from the interior of the cells, the so called "inside-out signalling", which converts the integrin from resting state to an activated state. The conformational change associated with cell binding leads to transduction of signals from the exterior to the interior of the cells, the so called "outside-in signalling", and to the regulation of cell functions such as proliferation, differentiation, migration and gene regulation [8].

Endothelial cells are located at the interface of the vascular system and are particularly exposed to reactive oxygen species (ROS) present in the blood stream, especially, the superoxide anion ($O^{\cdot}_2$), formed by the univalent reduction of oxygen; this reaction is mediated by several enzymes including NADPH-oxidases and xanthine oxidase. Reviews of Halliwell and Gutteridge [9], and Dröge [10], have shown that

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ROS are important cell mediators involved in cellular signalling or cell proliferation, and are thus essential for cell survival. The antioxidant defence mechanism maintains a balance between ROS production and scavenging, but an important increase in ROS production overcomes antioxidant defences leading to oxidative stress. Exposure of various cell types to oxidative stress conditions have been shown to trigger apoptosis [11–16]. In addition, in the absence of ECM interaction, endothelial cells rapidly undergo apoptosis a phenomena called anoïkis. Anoïkis as programmed cell death results from detachment of endothelial cells from ECM and this phenomenon has been reported to be associated with increased intracellular ROS [17].

Expression of several adhesion molecules in endothelial cells including ICAM-1, VCAM-1 is ROS dependent leading to monocyte adhesion and migration [18]. Beta-2 integrin was also reported to be modulated by oxidative stress in peripheral blood cells such as polymorphonuclear neutrophils and eosinophils [19]. Oxidative stress is frequently assumed to modulate cell to cell adhesion, but only little and contradictory information is available concerning the effects of oxidative stress on cell adhesion to the ECM. Oxidative stress inducer H_2O_2 is reported to decrease adhesion of mononuclear cells to collagen [20] and decreases adhesion of trabecular meshwork to fibronectin (Fn) [21]. We previously observed that cell treatment by *tert*-butyl hydroperoxide (*t*-BHP) which has been found to promote the lipid peroxidation, had opposite effects on cell adhesion to collagen depending on the cell type. Adhesion of ECV304 cells was decreased whereas that of EA.hy926 was increased, without modification of cell surface expression of α2 and β1 integrin subunits [22].

In this paper, emphasis was placed on the study of the relation between changes in endothelial cell adhesion to ECM and integrin expression in relation to apoptosis induced by oxidative stress. For this purpose, we investigated the effect of oxidative stress generated either by the hypoxanthine/xanthine oxidase system (HX/XO) or the direct chemical oxidant, *t*-BHP, on adhesion of EA.hy926 endothelial cells to vitronectin (Vn) and fibronectin (Fn) and on the cell surface expression of integrins and their consequences on apoptosis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, Hanks balanced salt solution (HBSS) and phosphate buffered saline (PBS) were from Gibco-BRL (InVitrogen, Cergy, France). Human plasma Fn was from Institut Jacques Boy (Reims, France), Human plasma Vn was from Calbiochem (VWR International, Fontenay sous Bois, France). Hypoxanthine (HX), xanthine oxidase (XO) (EC 1.1.3.2.2), *t*-BHP at 70% (v/v) in aqueous solution, α-D-tocopherol, and *N*-acetylcysteine were from Sigma Chemical Co. (St-Quentin-Fallavier, France). Calcein acetoxymethylester (calcein-AM), 2′,7′-dichloro-fluorescein diacetate (DCFH-DA), 5′-chloromethyl fluorescein diacetate (CMF-DA) and yopro-1 were from Molecular Probes (Eugene, OR). Mouse IgG and goat anti-mouse IgG-conjugated fluorothiocyanate fluorescein (FITC) were from Caltag (Burlingame, CA). Monoclonal antibodies directed against human integrin β1 (P4C10), α5 (P1D6) and αv (VNR 139) subunits from Life Technologies (Grand Rapids, NY,), α2 (P1E6), α3 (P1B5), from Becton Dickinson (Le Pont de Claix, France), β3 (25E11) from Chemicon Int. (Temecule, CA), αvβ3 (LM609) and αvβ5 (P1F6) from Caltag. Cellular DNA fragmentation ELISA kit was from Boehringer Mannheim (Meylan, France). Apoptosis detection kit (TACS™ annexin V-biotin) and isothiocyantae-conjugated streptavidin were from R&D Systems (Minneapolis, MN). Mowiol® was from Calbiochem (San Diego, CA). Polystyrene flasks and 96-well cell culture plates were from Falcon (Becton Dickinson, Le Pont de Claix, France).

Cell cultures

The endothelial cell line EA.hy926 [23] was provided by Dr L. Bordenave (University of Bordeaux, France) with permission of Dr C.J.S. Egdell (Department of Pathology, University of North Carolina, Chapell Hill, NC). Cells were plated at the initial density of 5×10^4 cells/ml and cultivated at 37°C under humidified atmosphere and 5% CO_2 in DMEM supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell viability was routinely measured using trypan blue exclusion test and cell populations with at least 90% viability were used for the experiments.

Induction of oxidative stress

Oxidative stress was induced using HX/XO or *t*-BHP. In the case of HX/XO treatment, freshly suspended cells were incubated in Hank's balanced salt solution (HBSS) containing HX (20 μg/ml) and XO at concentrations ranging from 2.5 to 20 mU/ml for various periods of time (30–90 min). The amounts of superoxide anion generated by the different HX/XO concentrations have been measured according to the cytochrome c reduction method [24] and the concentrations used corresponded to 2–18 nanomoles/30 min/ml for 2.5–20 mU/ml of XO, respectively. XO activity was systematically controlled according to

Rollet-Labelle et al. [12] by measuring H_2O_2 production using *o*-dianisidine (0.16 mM) as the substrate and peroxidase (1.8 U/ml) which catalyzes the oxidation of *o*-dianisidine by H_2O_2.

In the case of *t*-BHP treatment, freshly suspended cells were submitted to *t*-BHP (62.5–500 μM) for various periods of time (30–90 min) as already described [22].

Cell protection towards oxidative stress was obtained by pre-incubating the cells with α-D-tocopherol (200 μg/ml for 18 h) [25] or *N*-acetylcysteine (25 mM for 1 h) prior to apply oxidant treatments [26].

Measurement of ROS and thiol

Fluorescence microplate assay (FMA) was used to measure ROS production and thiol level as we previously described [26]. Briefly, treated and untreated cells were distributed in 96-well plates and stained with specific fluorogenic probes, 2′,7′-dichlorofluorescin diacetate (DCFHDA; 10 μM) and 5′-chloromethylfluorecein diacetate (CMF-DA; 5 μM) for ROS and thiol levels, respectively. After 30 min of incubation at 37°C, fluorescence intensity was measured at λex 485 nm and λem 538 nm (Fluostar, BMG LabTechnologies, Champigny-sur-Marne, France). The percentage of ROS production or reduced glutathione (GSH) depletion were expressed as the ratio of fluorescence intensity measured on treated and untreated cells. All measurements were done in triplicate.

Apoptosis assays

The percentage of apoptotic cells was measured by labeling cells with the DNA intercalant yopro-1 (2.5 μM) for 30 min at 4°C in the dark and reading fluorescence intensity at λ ex 485 nm and λem 538 nm as previously described [15]. Cellular DNA fragmentation assay was performed using the ELISA kit according to the manufacturer's instructions. Bound DNA fragments were detected by the addition of anti-BrdU-peroxidase monoclonal antibody (mAb). After substrate addition, optical density (OD) was determined with the Fluostar reader set at 370 nm. Externalization of phosphatidylserine occurring during apoptosis was followed by annexin-V staining [27] using apoptosis detection kit. For this purpose, EA.hy926 cells were cultured to subconfluency in slide chambers (labTek) and treated or not with oxidants, as described above. The cells were incubated with biotinylated-annexin-V for 15 min at room temperature, washed, incubated for 15 min at room emperature with FITC-conjugated streptavidine, washed three times with HBSS, and mounted in Mowiol® before examination with a microscope equipped with epifluorescence optics (Axiophot, Zeiss, Germany).

Cell adhesion assays

Cell adhesion assays were carried out as previously described [28] using the fluorogenic probe calcein-AM which entered the cells, and is cleaved into fluorescent marker by esterases of viable cells. Briefly, trypsinized EA.hy926 cells (2.5×10^5 cells/ml) were submitted or not to the oxidative treatment and labeled with calcein-AM (5 μM) for 30 min at 37°C. After rinsing with HBSS the cells were plated in 96-well plates (200 μl/well) uncoated or coated with Vn (100 ng/well) or Fn (20 μg/well). After incubation at 37°C for 30, 60 and 90 min, the plates were scanned in the Fluostar at λex 485 nm and λem 538 nm, gently rinsed with HBSS and scanned again. The relationship between the fluorescence intensity produced in cells by the calcein-AM and the cell number was linear over the range 2.5×10^4–8×10^5 cells/-well (least square regression, $r = 0.99$). Under these conditions, the values were in the range of 500–35,000 Arbitrary Fluorescence Units (AFU). The percentage of adherent cells was expressed as the ratio of the fluorescence intensity measured after and before washing. For inhibition experiments, trypsinized EA.hy926 cells were pre-incubated for 30 min at 37°C with monoclonal antibodies (diluted 1/50) against different integrin subunits prior to cell adhesion assays.

FACS analysis

Integrin expression was measured by flow cytometry analysis (FACS). Treated and untreated cells (106 cells/ml) were fixed with 4% paraformaldehyde for 15 min at room temperature and washed three times with PBS containing 3% bovine serum albumine (PBS–BSA). Cells were incubated for 60 min at room temperature with mouse monoclonal antibodies (diluted 1/100) directed against human integrin chains. After three washes with PBS–BSA, the cells were incubated for 45 min in the presence of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (diluted 1/100). The cells were washed 3 times with PBS–BSA, resuspended in 500 μl PBS–BSA and fluorescence analysis was performed by flow cytometry with Coulter Elite ESP flow cytometer (Beckman-Coulter, Roissy, France) using a 15 mV air-cooled argon–ion laser set at 488 nm. In control experiments, the primary antibody was omitted and cells were incubated with the secondary FITC-conjugated antibody alone. For each measurement, data were collected from 5000 to 10,000 cells. Data analysis was performed with standard spreadsheet software and results expressed as the mean fluorescence intensity (MFI).

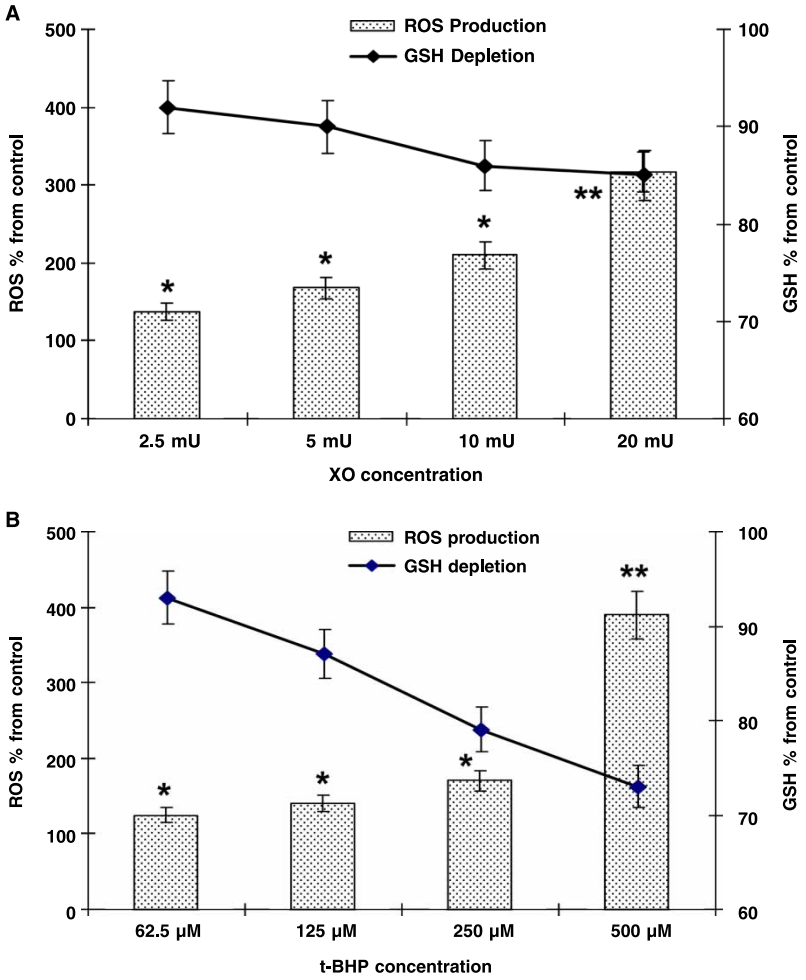


Figure 1. HX/XO or *t*-BHP treatment induces ROS production and GSH depletion in EA.hy926 cells. ROS production was measured using DCFH-DA and GSH depletion using CMF-DA as described in materials and methods. (A) Cells were treated for 60 min with increasing XO concentrations. (B) Cells were treated for 60 min with increasing *t*-BHP concentrations. The values are expressed as the percentage increase (ROS) or decrease (GSH) compared to control (untreated cells).

Statistical analysis

Results were expressed in values calculated from three independent experiments carried out in triplicate. Results are presented as mean ± standard error (SE). Significant differences were analyzed by Student's *t*-test. Values of p inferior to 0.05 were considered statistically significant.

Results

Oxidant treatments enhance ROS production and induce apoptosis in EA.hy926 cells

Oxidative stress was induced in endothelial EA.hy926 cells by HX/XO or *t*-BHP treatments and monitored by specific measurement of ROS and thiol levels: both treatments resulted in a time- (not shown) and concentration-dependent increase of ROS production (Figure 1A, B). A treatment with 2.5–20 mU/ml of XO resulted in a 1.3–3-fold increase ROS, and a treatment with 62.5–500 µM of *t*-BHP resulted in a 1.2–4-fold increase of ROS. Concomitantly to ROS production, XO or *t*-BHP treatments induced a slight time- and concentration-dependent thiol depletion and did not exceed 15% ($p < 0.05$) or 25% ($p < 0.05$) respectively (Figure 1A, B). To verify whether ROS production was specifically induced by the oxidant systems EA.hy 926 cells were incubated prior to the treatments with α-D-tocopherol [25], or *N*-acetylcysteine [26], known to protect against oxidative stress. Under these conditions, with α-D-tocopherol there were only a 20% increase in ROS production in the case of XO and *t*-BHP treatments, compared to a 100% increase in unprotected cells ($p < 0.01$). Performing pre-treatment with *N*-acetylcysteine, ROS production is partially prevented after XO or *t*-BHP treatments by 40% ($p < 0.01$) respectively.

In addition to enhanced ROS production, both treatments induced apoptosis in a concentration- and time-dependent manner (Figure 2A1, A2, B1, B2, C, D1, D2). Apoptosis using Yopro-1 labeling which

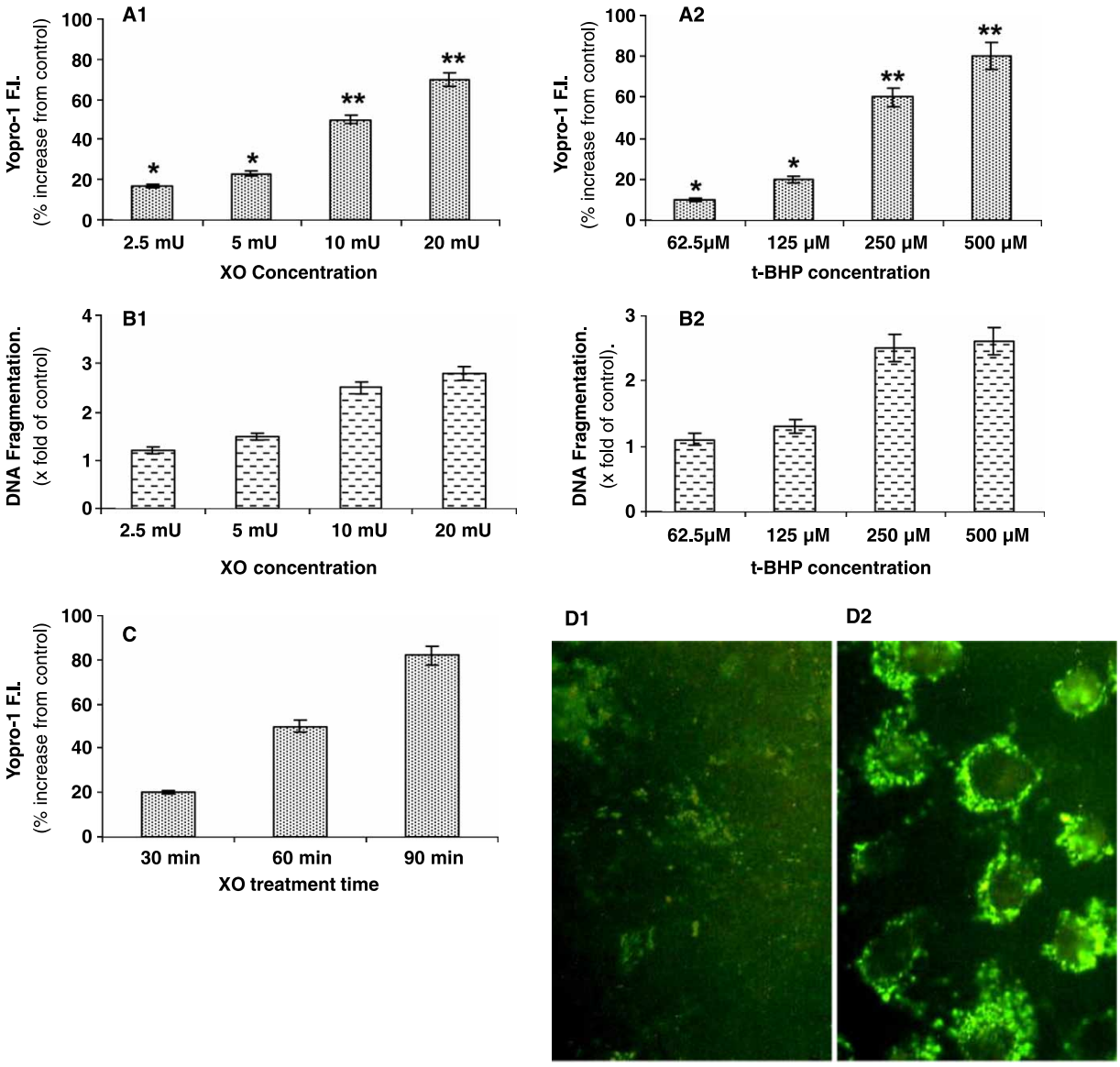


Figure 2. HX/XO or *t*-BHP treatment induces apoptosis in EA.hy926 cells. Cells were treated for 60 min with increasing XO concentrations (A1 and B1) or increasing *t*-BHP concentrations (A2 and B2) or with 10 mU/ml XO for different periods of time (C). (A1), (A2) and (C) Apoptosis was measured using yopro-1 as described in materials and methods. Results are given in percentage increase compared to control (untreated cells). (B1) and (B2) Apoptosis was measured by DNA fragmentation using anti-BrdU-peroxidase mAb. Results are expressed in x-fold OD increase compared to control (untreated cells). Values in A, B and C represent mean ± SE from three different experiments performed in triplicate. Dapoptosis was evidenced by annexin-V staining. Cells were cultured at subconfluence in Labtek, untreated (D1) or treated (D2) with XO (10 mU, 60 min), and processed for immunofluorescence of Annexin-V as described in materials and methods.

revealed changes in membrane permeability, as it occurs in apoptotic cells [29], was increased by 50 or 60% ($p < 0.01$) following 60 min treatment with 10 mU/ml XO or 250 µM *t*-BHP, respectively (Figure 2A1,A2). Specific detection of cellular DNA fragmentation showed a 2.5-fold increase in cells treated with 10 mU/ml XO or 250 µM *t*-BHP compared to untreated cells (Figure 2B1,B2). These results were confirmed by a distinct annexin-V staining of the plasma membrane of XO-treated cells, whereas untreated cells exhibited only a weak staining of the plasma membrane (Figure 2D1,D2). Moreover, ROS-induced apoptosis was reduced by 50 or 30%, respectively, by α-D-tocopherol or *N*-acetylcysteine pretreatment, compared to unprotected cells (data not shown).

Cell adhesion to extracellular matrix prevents ROS-induced apoptosis

In the above experiments, ROS induction and subsequent apoptosis were observed in cells plated on polystyrene tissue culture plates. As adhesion to ECM protects cells against apoptosis, or anoikis [30], we next tested whether adhesion of EA.hy926 cells to Vn and Fn could antagonize ROS-induced apoptosis.

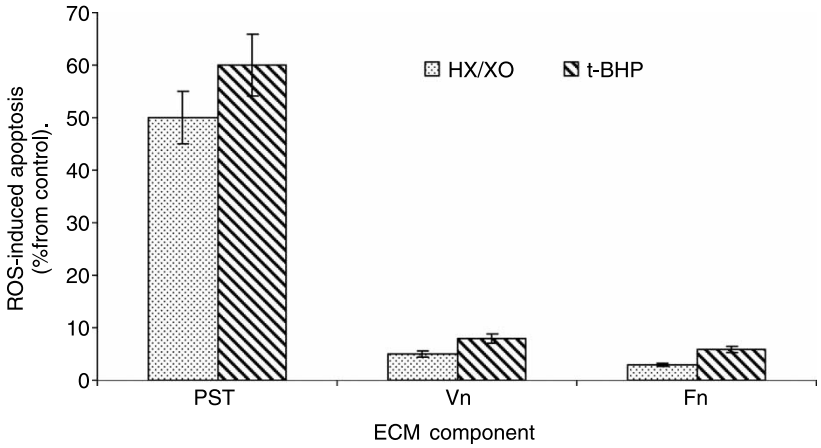


Figure 3. Apoptosis triggering inhibition by ECM components. Cells were treated by HX/XO (10 mU/ml XO) or by *t*-BHP (250 μM) for 30 min and plated on 96 well microplates pre-coated with Fn (20 μg/well) or Vn (100 ng/well), incubated for 60 min at 37°C, and apoptosis was measured using yopro-1 as described for Figure 2A. Values represent mean ± SE from three different experiments performed in triplicate.

XO or *t*-BHP-treated cells were plated onto Vn and Fn, and apoptosis was measured after 60 min of adhesion to the coated proteins. There was nearly no apoptosis in XO (3–5%) or *t*-BHP (5–8%) treated cells adhering onto Vn and Fn compared to 50 or 60% for cells plated on uncoated plates, respectively (Figure 3). This indicated that plating cells onto Vn and Fn allows a 95–85% protection against apoptosis according to XO or *t*-BHP treatment, respectively. The Vn or Fn apoptosis protection was inhibited in the presence of anti-αv and anti-α5 blocking monoclonal antibodies, since apoptosis of XO-treated cells reached 40% in the presence of anti-αv on Vn, and 60% in the presence of anti-α5 on Fn.

Oxidative stress modulates cell adhesion to Vn and Fn and integrin expression

We subsequently examined the adhesion properties of oxidant-treated cells. EA.hy926 cells treated with increasing XO or *t*-BHP concentrations were plated on Vn or Fn coats and the extent of adhesion was measured after 60 min adhesion period (Figure 4A1, A2). At low XO (2.5–5 mU/ml) or *t*-BHP (62.5–125 μM) concentrations, cell adhesion to both Vn and Fn was reproducibly increased (up to 25%, $p < 0.05$). In contrast, at higher XO (10 mU/ml) or *t*-BHP (250 μM) concentrations, a decrease of cell adhesion to Vn and Fn was observed, and reached 20 and 40%, respectively ($p < 0.05$), this decrease was low at 30 min (Figure 4B) and best seen after 90 min (Figure 4C). Thus oxidative stress has a bimodal effect on the adhesion of EA.hy926 cells to the ECM, low and high concentrations of oxidants inducing an increase and a decrease in cell adhesion, respectively. The same bimodal effect of oxidant treatment was observed after plating the cells on Vn and Fn for 30 and 90 min cell adhesion, as shown for XO treatment (Figure 4B, C).

Cell adhesion to ECM proteins is known to involve cell surface receptors of the integrin family. In particular, adhesion to Vn is mediated by αv-containing integrins and adhesion to Fn is mainly mediated by α5β1 integrin [31]. We therefore analyzed integrin expression on the surface of untreated (control) and oxidant-treated EA.hy926 cells by flow cytometry. In control cells, analysis of single integrin subunits showed expression of β1, α5, β3, and αv Figure 5A1, A2 and analysis of specific detection of heterodimers indicated the presence of integrins αvβ3 and αvβ5 (Figure 5B). Expression of integrin α2 and α3 subunits was also observed and not affected by oxidant treatment (not shown).

Oxidant treatments revealed modifications in the α5 and αv integrin patterns of the cells. In XO (5 mU/ml, 30 min) or *t*-BHP (125 μM, 30 min) treated cells, the level of integrin αv subunit was increased while integrin α5 was decreased and the levels of β1 and β3 were unchanged (Figure 5A1, A2). Analysis of the expression of the two integrins sharing the same αv subunit (αvβ3 and αvβ5) using complex specific mAb showed slight modifications of αvβ3 and αvβ5 expression after XO treatment (Figure 5B). Thus, the main changes concerned αv and α5 expression and α-D-tocopherol pre-treatment of oxidant-treated cells prevented partially the increase in αv expression and totally the change in α5 expression (Figure 6).

Integrins involved in cell adhesion to Vn and Fn

To determine which integrins were involved in adhesion of oxidant-treated and untreated cells to Vn and Fn, inhibition assays were performed using function-blocking antibodies directed against integrin subunits and results are presented in Table I.

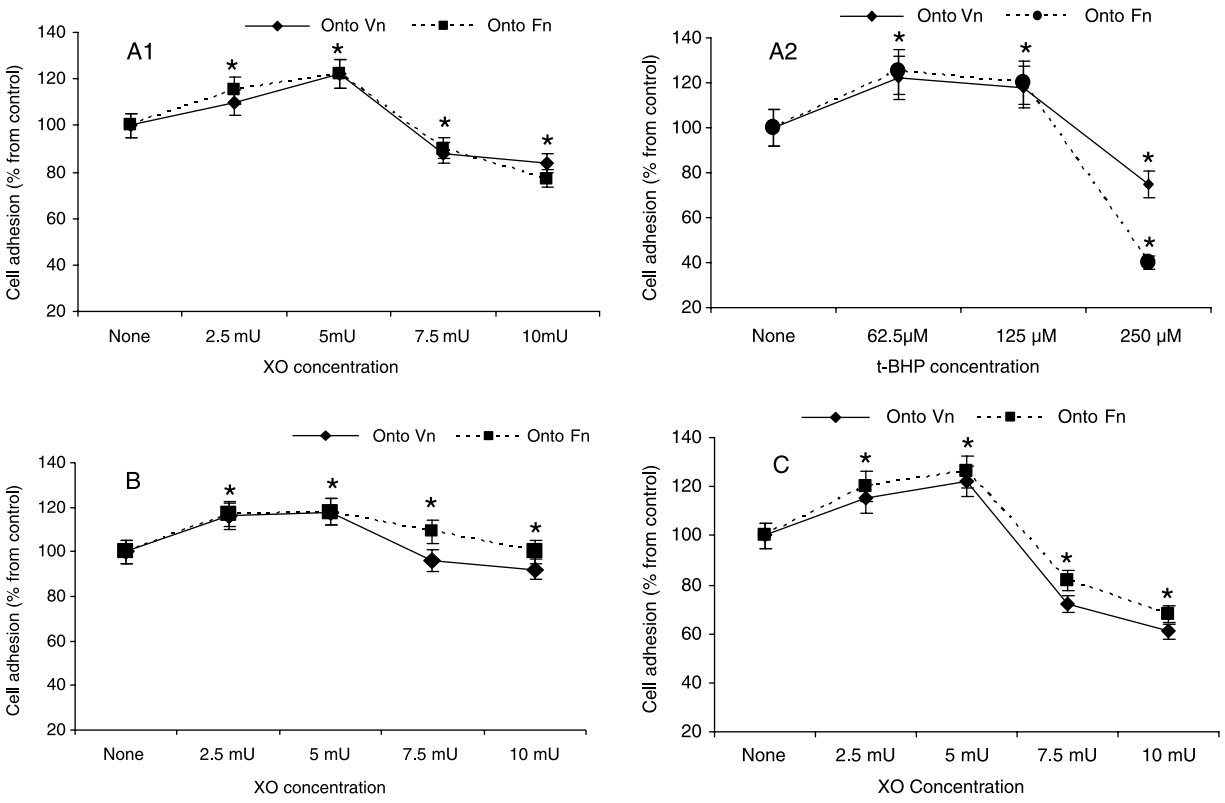


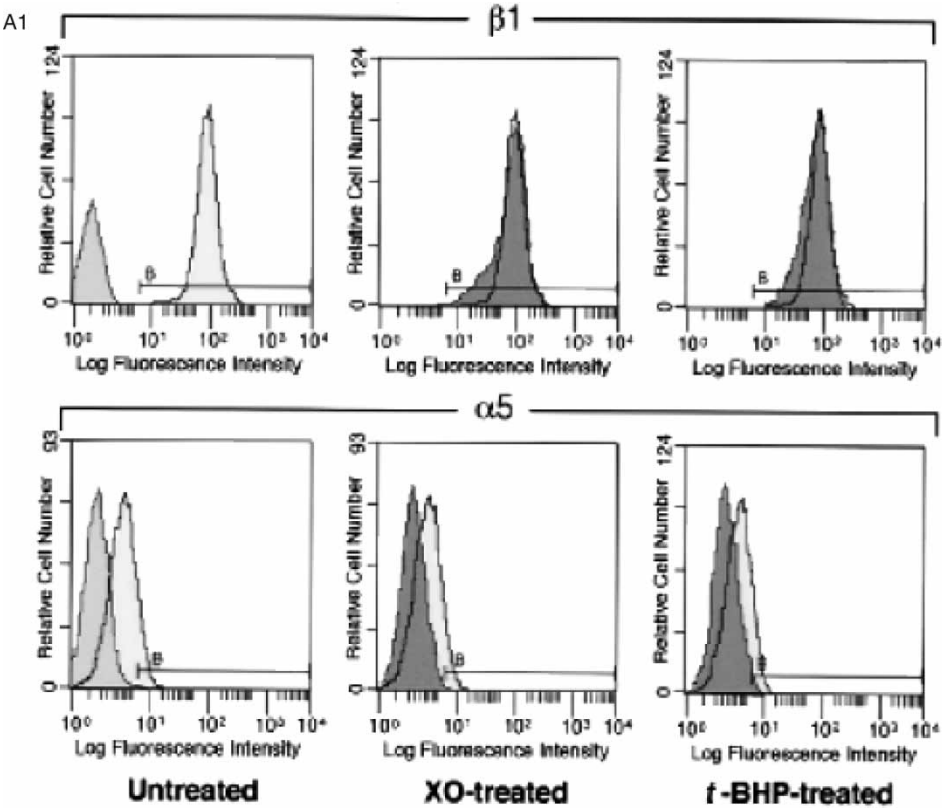
Figure 4. Effects of oxidative stress on cell adhesion on Vn or Fn. Cell adhesion assays were carried out as previously described [28] using the fluorogenic probe calcein-AM which entered the cells, and is cleaved into fluorescent marker by esterases of viable cells. EA.hy 926 cells (2.5×10^5 cells) were labeled with calcein-AM (5 μM, 30 min), treated or not with increasing XO (A1) or *t*-BHP (A2) concentrations, as described in Material and Methods, plated onto Vn- or Fn-coated 96 well microplates, and incubated for 60 min at 37°C. Cell adhesion of XO-treated cells was also measured after 30 min (B) and 90 min (C) incubation at 37°C. Results were expressed as percentages of adherent cells. Values are means ± SE from three different experiments done in triplicate.

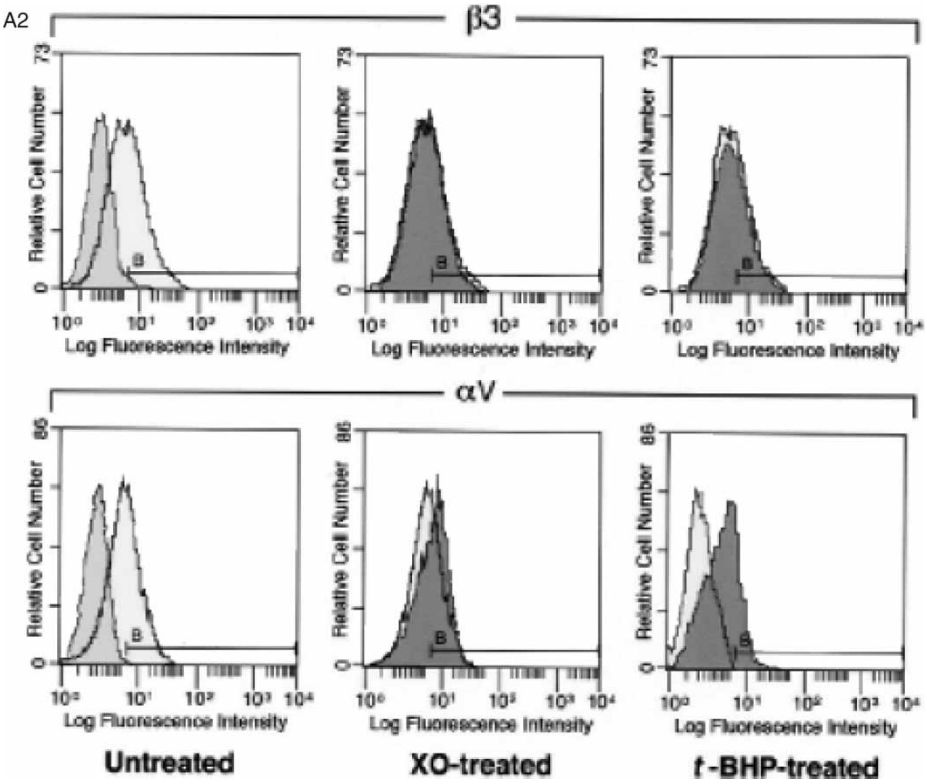
In the presence of mAb directed against integrin β1 subunit, a 25% inhibition was observed for untreated cells adhering to Fn, while a 58 or 55% inhibition was observed for XO or *t*-BHP-treated cells, respectively. Adhesion of untreated and XO or *t*-BHP-treated cells to Vn was found to be inhibited by mAb against integrin β1 subunit to comparable extents 42, 38 and 35%, respectively. Adhesion to Fn of untreated and XO or *t*-BHP-treated cells was decreased by 10 and 25 or 22%, respectively, in the presence of mAb against integrin β3 subunit. Similarly, adhesion of untreated and XO or *t*-BHP- treated cells to Vn was decreased by 20 and 33 or 30% by mAb against integrin β3 subunit, respectively. In contrast, mAb against integrin α5 subunit inhibited ≥50% adhesion of both untreated and oxidant-treated cells to Fn while it did not, or slightly (17–14%) inhibited adhesion to Vn. Finally mAb against integrin αv subunit inhibited adhesion of XO- treated cells to both Vn and Fn by 58 and 50%, respectively, while it reached 30 and 10% in the case of adhesion of untreated cells to Vn and Fn. In the case of *t*-BHP treatment, inhibition of adhesion to Vn or Fn reached 55 and 45%, respectively.

Taken together, the results showed that XO as well as *t*-BHP treatments modified the adhesion to Vn and Fn matrices. So, ROS generated either by HX/XO or *t*-BHP could affect expression and/or activation of specific integrins in the interaction of EA.hy926 cells with ECM.

Discussion

In this paper, we show that experimentally induced oxidative stress in EA.hy926 cells affects αv and α5 integrin expression resulting in modifications in cell adhesion. The implication of ROS in these processes was confirmed by the use of α-D-tocopherol and *N*-acetylcysteine as antioxidant agents. Moreover, low XO (≤5 mU/ml) or *t*-BHP (≤125 μM) and high XO (≥10 mU/ml) or *t*-BHP (≥250 μM) concentrations had opposite effects on the cell adhesion onto the ECM proteins, Vn and Fn. Cell treatment with high XO or *t*-BHP concentrations resulted in high ROS level and decreased cell adhesion to Vn and Fn. Increasing the time of cell adhesion to 60 or 90 min led to enhancement of the effect of high XO concentrations. On the contrary, treatment of EA.hy926 cells with low XO or *t*-BHP concentrations resulted in a weaker, albeit distinct, ROS production and increased cell adhesion to Vn and Fn. The threshold





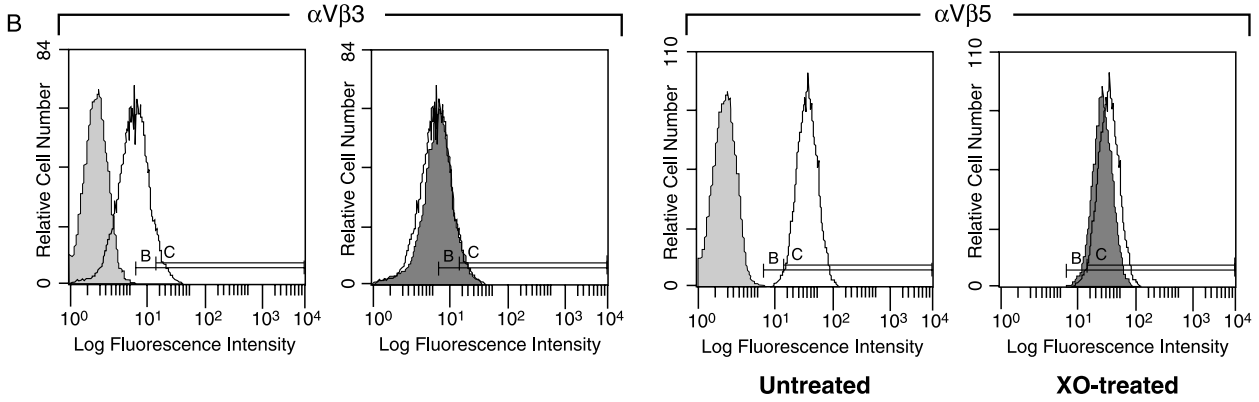


Figure 5. Integrin expression in control and oxidant-treated cells. Integrin expression was determined by FACS analysis as described in material and methods before and after oxidant-treatment. (A1) β1 and α5 integrin subunit expression, Grey peak, non-immun FITC; black open line, untreated cells; dark grey peak, XO or *t*-BHP-treated cells. (A2) β3 and αv integrin subunit expression, Grey peak, non-immun FITC; black open line, untreated cells; dark grey peak, XO or *t*-BHP-treated cells. (B) αvβ3 and αvβ5 integrin expression. Grey peak, non-immun FITC; black open line, untreated cells; dark grey peak, XO-treated cells.

of the bimodal effect observed on cell adhesion could be set between 5 and 7 nanomoles/ml of superoxide anions. Such biphasic effect has been described recently by Huang and Zheng [32]: migration and adhesion of endothelial cells was enhanced with low concentration of O_2- generated by 1 mU/ml but significantly inhibited as the XO increased to 10 mU/ml. Besides, Batelli et al. [33] showed that apoptosis of human peripheral blood lymphocytes is mostly depending on XO concentration.

Along the same lines, an upregulation of VCAM-1, a cell surface counter-receptor for integrins, was previously observed in endothelial cells submitted to oxidative stress produced by IL-4 treatment [34]. Chemically induced oxidative stress has been reported to modify cell–cell interactions by disrupting the cadherin/catenin complex [35]. It has been suggested that high ROS level results in lipid peroxidation and thus affects membrane fluidity as shown in erythrocytes [36]. Integrins are transmembrane receptors which ligand binding function is regulated by exposure at the cell surface and clustering [37]. Therefore, ROS-induced modification of membrane fluidity could affect integrin exposure at the cell

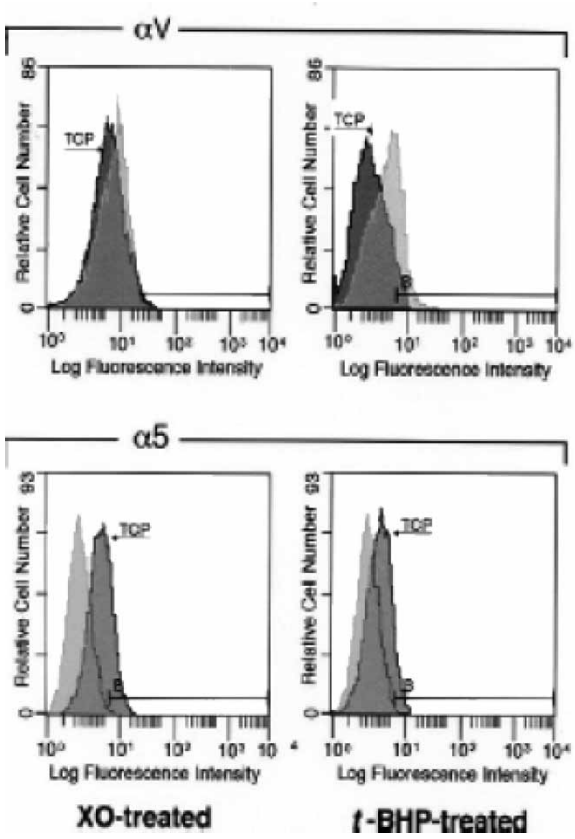


Figure 6. α-D-tocopherol protects cells against effects of oxidative stress. Cells were incubated (or not) for 18 h with 200 μg/ml of α-D-tocopherol (TCP) before XO- (5 mU/ml for 30 min) or *t*-BHP- (125 μM for 30 min) treatment and expression of αv and α5 integrin subunits was analyzed by FACS as described in material and methods. Grey peak, oxidant-treated cells; black peak, α-D-tocopherol pre-treated cells before oxidant-treatment.

Table I. Adhesion of E.A.hy 926 cells onto Fn and Vn is differently inhibited by MoAb directed against β1-, β3-, α5- and αv-integrin subunits*.

	Fibronection			Vitronection		
MoAb	Untreated cells	XO-treated cells	*t*-BHP treated cells	Untreated cells	XO-treated cells	*t*-BHP treated cells
Anti-β1	75	42	45	58	62	65
Anti-β3	90	75	78	80	67	70
Anti-α5	50	46	54	100	83	85
Anti-αv	90	50	55	70	42	45

* E.A.hy 926 cells were preincubated with the indicated MoAb prior to the cell-adhesion assay, as described in *material and methods*. Each value is the mean of those obtained from three separate experiments run in triplicate, and SD did not exceed 10%. The amount of adherent cells was quantitated as described in *material and methods*. The adhesion in the presence of MoAb is expressed as the percentage relative of the adhesion of untreated or XO or *t*-BHP-treated cells in the absence of the MoAb (arbitrarily set at 100% for each case).

surface and/or alter their clustering explaining the changes in cell adhesion observed after oxidant treatment. Alternatively, low ROS production has been known to activate different signalling cascades [38]. Chiarugi et al. [39] have related important observations on the role of ROS as physiological regulators of tyrosine kinase receptor signalling cascades and have shed new lights on the possible mechanisms underlying the activity of oxidants agents. Moreover, the oxidant treatments have been correlated to tyrosine kinase signal transduction linked to adhesion capacity and integrin receptor signaling [40] and thus may lead to an increase in cell surface expression and/or activity of cell adhesion molecules involved in cell–ECM interactions. We previously observed such tyrosine kinase dependent mechanisms in the increase of EA.hy 926 cell adhesion to collagen induced by *t*-BHP treatment using genistein pre-treatment [22]. The increase in cell adhesion to Vn and Fn of XO or *t*BHP-treated cells could be attributable to changes in the cell surface integrin expression involved in this process. To test this possibility, we analysed by FACS differential modulations in integrin expression and the major findings are: (i) increase of αv, (ii) decrease of α5 and (iii) no change of β1 or β3 integrin subunit expression. The increase of αv integrin subunit was in line with the increase of cell adhesion to Vn and was in agreement with the role of ROS in the signaling cascade mediated by integrins during cell–ECM interactions as reported by Chiarugi et al. [39]. Concerning the decrease of the α5 integrin subunit and the increase of cell adhesion to Fn observed in parallel, a direct role of ROS on the α5 molecule could be suggested, leading to alteration in its conformation and thus of its activity [31]. Thus the integrin α5 subunit could be activated by ROS production, as it was recently reported for the β3 subunit [41]. It was shown that integrin was a direct target for redox modulation and that integrin activation could be controlled directly by a redox site in the extracellular domain independently from cytoplasmic factors.

Cell adhesion inhibition assays, using specific function-blocking monoclonal antibodies confirmed the involvement of αv, α5, β1 and β3 integrin subunits in cell adhesion to Vn and Fn. In addition, our results indicated that in untreated cells both αvβ1 and αvβ3 integrins were involved in adhesion to Vn, whereas adhesion of XO- or *t*-BHP-treated cells depended mainly on αvβ3 integrin. In the case of cell adhesion to Fn, untreated cells used mainly α5β1 and XO- or *t*-BHP -treated cells used both αvβ1 and α5β1.

Moreover, the involvement of cell surface adhesion molecules in apoptosis triggering after XO or *t*-BHP treatments was observed. The interaction of these molecules with Vn or Fn was found to be able to protect cells against apoptosis whereas this effect is lost in the presence of blocking mAb. These results are in line with those reported by Nisato et al. [6] concerning αvβ3 and αvβ5. In addition, the results are in accordance with already published data [22] concerning adhesion of EA.hy926 cells onto collagen that showed an increase of adhesion without increase of expression of the integrins α2 and β1. In line with the growing attention towards the mechanisms of intracellular signalling by ROS, results of the present study indicated that ROS play a role in endothelial cells–ECM interactions by modulating integrin expression and/or activation. Moreover, the increase in cell adhesion led to the protection of cells against ROS-induced apoptosis. Such a protection against H_2O_2-induced apoptosis was also observed in U 87 cells by the extracellular proteoglycan versican as reported by Wu et al. [42].

In conclusion, using EA.hy926 cell model our results showed that (1) cell adhesion to Vn or Fn prevent ROS-induced apoptosis, (2) ROS production modulated integrin expression and/or state of activity, and also cell adhesion to Vn or Fn. With respect to cell survival, ROS is in general associated with induction of apoptosis. However, under certain conditions such as oxidant concentration, [43] and as reported in the present paper, ROS increasing cell adhesion can also be anti-apoptotic. Thus, modulation of integrin expression and function by ROS could be part of the cell strategy aimed at increasing cell survival.

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