Activated neutrophils oxidize extracellular proteins of endothelial cells in culture: effect of nitric oxide donors

Grzegorz A. CZAPSKI*[1], Diana AVRAM†‡, Dmitri V. SAKHAROV‡, Karel W. A. WIRTZ‡, Joanna B. STROSZNAJDER* and Everard H. W. PAP‡[2]

*Department of Cellular Signalling, Medical Research Centre, Polish Academy of Sciences, Pawinskiego 5, 02-106 Warsaw, Poland, †Department of Immunology, Cantacuzino Institute, Splaiul Independentei 103, 70100 Bucharest, Romania, and ‡Department of Biochemistry of Lipids, Institute of Biomembranes, Utrecht University, Padualaan 8, NL-3584 CH, Utrecht, The Netherlands

Protein oxidation of human umbilical-vein endothelial cells (HUVEC) in culture was examined under various conditions of oxidative stress. Extracellular protein (ECP) oxidation was assessed by determining dityrosine bond formation, which is indicated by the covalent coupling of the membrane-impermeable tyramine–fluorescein conjugate (TyrFluo) to HUVEC proteins. The acetylated membrane-permeable form of TyrFluo (acetyl-TyrFluo) was used for the determination of intracellular protein (ICP) oxidation. Oxidative stress was induced by exposing the HUVEC to PMA-activated human neutrophils, to a horseradish peroxidase/hydrogen peroxide (HRP/H_2O_2) system or to H_2O_2 alone. Coupling of the probes was determined by confocal laser scanning microscopy and by Western blotting using anti-fluorescein antibody. Diethylamine nitric oxide (DEANO) was used to determine the effect of NO on the tyrosyl radical formation in proteins. The oxidative burst generated by activated neutrophils for 15 min, resulted in inducing dityrosine formation in ECP of HUVEC. Similar results were obtained with HRP/H_2O_2, but H_2O_2 alone did not have any effect on ECP. In the presence of DEANO (0.1 mM or higher), ECP oxidation was almost completely inhibited. This indicates that NO may protect endothelial cells against protein oxidation by activated neutrophils under pro-inflammatory conditions. Activated neutrophils did not oxidize ICP of HUVEC, which strongly suggests that the effect of the oxidative burst was restricted to the proteins exposed to the medium.

Key words: dityrosine, fluorescent probe, oxidative stress, protein oxidation, HUVEC.

INTRODUCTION

Endothelial cells play an important role in the protection against infectious agents as well as in the transport of electrolytes and nutrients. The endothelium regulates the blood flow and vasomotor tone and also produces antithrombotic factors and adhesion molecules. The endothelial cells control the vascular resistance by synthesizing and releasing vasoactive mediators such as NO, prostacyclin and endothelins [1,2]. Acetylcholine and bradykinin induce the production of NO by the endothelial isoform of NO synthase [3–5].

Activated neutrophils, as a consequence of an inflammatory response, generate free radicals to destroy bacterial invaders. During oxidative burst they release high amounts of reactive oxygen species, proteolytic enzymes and pro-inflammatory cytokines [6,7]. Overactivation of neutrophils in many pathological states results in the damage of the endothelium. Oxidative agents produced by neutrophils, like myeloperoxidase (MPO), H_2O_2, superoxide anion ($O_2^{\cdot-}$), hypochlorous acid and peroxynitrite, can damage proteins, lipids and DNA [8]. An important protein modification by free radicals is the oxidative conversion of L-tyrosine into the tyrosyl radical, which can react with another tyrosyl radical to form dityrosine or with nitrogen dioxide ($^{\cdot}NO_2$) to form nitrotyrosine [9]. The formation of nitrotyrosine and dityrosine leads to an alteration in the protein structure and function. As a consequence, the activity of many cellular enzymes may be lost and phosphotyrosine-mediated signalling pathways may be impaired [10,11]. Protein tyrosine oxidation has important implications for functioning of the endothelium. Recently [12,13], a novel fluorescent probe, denoted as tyramine–fluorescein conjugate (TyrFluo), was developed for determining the protein oxidation in intact cells. This membrane-impermeable probe forms dityrosine bonds with oxidized extracellular proteins (ECPs). The acetylated form (acetylTyrFluo) is membrane-permeant and capable of forming dityrosine bonds with intracellular proteins (ICPs). Using these fluorescently labelled tyramine conjugates, we have determined whether (1) PMA-activated neutrophils can induce ECP and ICP oxidation of endothelial cells in culture and (2) an NO donor such as diethylamine nitric oxide (DEANO) has an effect on dityrosine formation in human umbilical-vein endothelial cells (HUVEC).

EXPERIMENTAL

Materials

Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution, tyramine, L-tyrosine, horseradish peroxidase (HRP), bovine liver catalase,

Abbreviations used: CLSM, confocal laser scanning microscopy; DEANO, diethylamine nitric oxide; ECP, extracellular protein; HRP, horseradish peroxidase; HUVEC, human umbilical-vein endothelial cells; ICP, intracellular protein; MPO, myeloperoxidase; TBS, Tris-buffered saline; TyrFluo, tyramine–fluorescein conjugate; acetylTyrFluo, acetylated membrane-permeant form of TyrFluo.

[1] To whom correspondence should be addressed (e-mail grzegorz@cmdik.pan.pl).

[2] Present address: Aventis Pharma AG, Drug Innovation and Approval/HTS, Industriepark Hoechst Bldg H811, D-65926 Frankfurt am Main, Germany.

PMA and Histopaque 1083 were obtained from Sigma. Sodium salt of DEANO along with succinimidyl ester of 6-(fluorescein-5-carboxamido)hexanoic acid and 6-(fluorescein-6-carboxamido)-hexanoic acid were from Molecular Probes (Europe BV, Leiden, The Netherlands). Acetic anhydride and 4-(dimethylamino)-pyridine were purchased from Merck (Frankfurt, Germany). Polyclonal anti-fluorescein antibody labelled with HRP was purchased from Biogenesis (Poole, Dorset, U.K.). Cell culture reagents were obtained from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.).

Cell culture

HUVEC were obtained from the Department of Hematology, Utrecht Medical Centre, Utrecht University, The Netherlands. HUVEC were grown in human endothelial serum-free medium system supplemented with 5% (v/v) heat-inactivated foetal calf serum, fibronectin, human recombinant epidermal growth factor and human recombinant basic fibroblast growth factor at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The medium was replaced daily by a fresh one, but a day before the experiment serum-free medium was used. Cells grown in 6-well plates with density of 60–80% were used for further experiments.

Preparation of polymorphonuclear neutrophils

Fresh blood from healthy volunteers was mixed with 1 vol. of Hanks buffer. It was then carefully placed on a Histopaque 1083 cushion and centrifuged at 1450 rev./min for 40 min at room temperature. Red blood cells in the pellet containing neutrophils were lysed for 30 min at 4 °C with 15 vols. of NH_4Cl (155 mM) and then the neutrophils were sedimented by centrifugation at 1200 rev./min for 15 min at 4 °C, and washed twice with Hanks buffer.

Synthesis of the fluorescein-tyrosine probe

The TyrFluo used for the detection of tyrosine oxidation of extracellular matrix proteins was prepared as described by van der Vlies et al. [12]. Briefly, the succinimidyl ester of 6-(fluorescein-5-carboxamido)hexanoic acid and 6-(fluorescein-6-carboxamido)hexanoic acid (1 mg) was coupled with the amine group of tyramine (3 mg) by incubating for 2 h in methanol supplemented with Bicine (200 mM) at room temperature, pH 8.5. After the reaction, TyrFluo was extracted from the incubation mixture by adding chloroform/methanol/25% ammonia in the ratio 4:2:1 (by vol.). TyrFluo partitioned in the chloroform phase, whereas unreacted tyramine was dissolved in the water phase. After vacuum-drying in a Rotofapor-R, TyrFluo was dissolved in PBS.

For the analysis of proteins that undergo tyrosylation reactions inside the cell, acetylTyrFluo was used. Acetylation of TyrFluo (10 mM) was performed in 50 μl of tetrahydrofurane in the presence of 0.2 mg of 4-(dimethylamino)pyridine and 3 μl of acetic anhydride. A colourless solution indicated that the acetylation was successful. Ethanol was added to react with the excess of acetic anhydride. The concentration of TyrFluo was determined spectrophotometrically using $\epsilon_{495} = 74000\ M^{-1} \cdot cm^{-1}$. Both TyrFluo and acetylTyrFluo were stored at −80 °C until use.

Determination of ECP and ICP oxidation

For the determination of ECP oxidation, HUVEC grown on 6-well plates were washed twice in PBS and incubated in Hanks buffer supplemented with 6 μM TyrFluo, 10 mM Tris (pH 7.5) and protease inhibitors, for 15 min at 37 °C. During the incubation, HUVEC (density 60–80%) were subjected to oxidative stress in three different ways: (1) with neutrophils (3.3×10^5 cells/ml) activated by PMA (2 μg/ml), (2) with H_2O_2 (100 μM) and (3) with HRP/H_2O_2 (4 munits/ml HRP and 10 μM H_2O_2). The culture plate was gently shaken to prevent the adhesion of neutrophils to HUVEC. In some experiments, DEANO (0–1 mM) was used as NO donor. After incubation, the medium was removed and the endothelial cells were washed twice in PBS to remove all the reagents and neutrophils. Proteins were collected from the cells lysed by the addition of ice-cold SDS-sample buffer. Proteins were denatured by heating at 95 °C for 5 min, then separated by SDS/PAGE (10% gel), and transferred on to a nitrocellulose membrane using a semi-dry system (LKB-Pharmacia, Uppsala, Sweden). After blocking [2×10 min with 0.2% non-fat milk and 2×20 min with 2% non-fat milk in Tris-buffered saline (TBS)-Tween], TyrFluo-labelled proteins were detected by incubating for 1 h with HRP-conjugated polyclonal antibody raised against fluorescein (diluted 1:1500 with 2% non-fat milk in TBS-Tween). Unbound antibody was removed by washing with TBS-Tween containing 2 and 0.2% non-fat milk for 5 and 3×10 min respectively, and also without non-fat milk for 5 min.

For the determination of ICP oxidation, cells were washed twice in PBS and then loaded with acetylTyrFluo (6 μM) in Hanks buffer supplemented with 10 mM Tris/HCl, pH 7.5, at 37 °C for 5 min. After removing the excess probe by washing twice in PBS, the cells were subjected to oxidative stress as described above. Under conditions where cells were stressed with H_2O_2 (100 μM) and with HRP/H_2O_2 (4 munits/ml HRP and 100 μM H_2O_2), catalase (250 units/ml) was added to the medium as a control.

TyrFluo bound to HUVEC proteins were revealed by confocal laser scanning microscopy (CLSM; Leica Microsystems, Heidelberg, Germany).

Determination of exogenous L-tyrosine oxidation

L-tyrosine (0.5 mM) was incubated in Hanks buffer at 37 °C with gentle mixing. Oxidative stress was induced by neutrophils (3.3×10^5 cells/ml) activated by PMA (2 μg/ml). DEANO (0–0.1 mM) was used to test the inhibitory effect of NO on dityrosine formation. During incubation, dityrosine formation was determined by the measurement of fluorescence on an SLM-AmincoSPF-500C spectrofluorimeter (λ_{Ex}, 325; λ_{Em}, 405).

RESULTS

ECP oxidation in endothelial cells

As shown by CLSM, the incubation of HUVEC with membrane-impermeable TyrFluo did not result in labelling under control conditions (Figure 1A). After 15 min of incubation with activated neutrophils, TyrFluo labelling of HUVEC was observed (Figure 1B). Similar results were obtained when the cells were exposed to oxidative stress induced by HRP/H_2O_2 (Figure 1C).

Dityrosine bond formation in HUVEC was determined by the detection of TyrFluo-labelled proteins by Western blotting (Figure 2). A limited protein oxidation pattern was observed when cells were incubated under control conditions (Figure 2B) and in the presence of non-activated neutrophils (Figure 2C). However, when the endothelial cells were incubated with PMA-activated neutrophils, an extensive oxidation of ECP was detected (Figure 2D). Similar results were obtained when the cells were exposed to oxidative stress with 4 munits/ml HRP and 10 μM

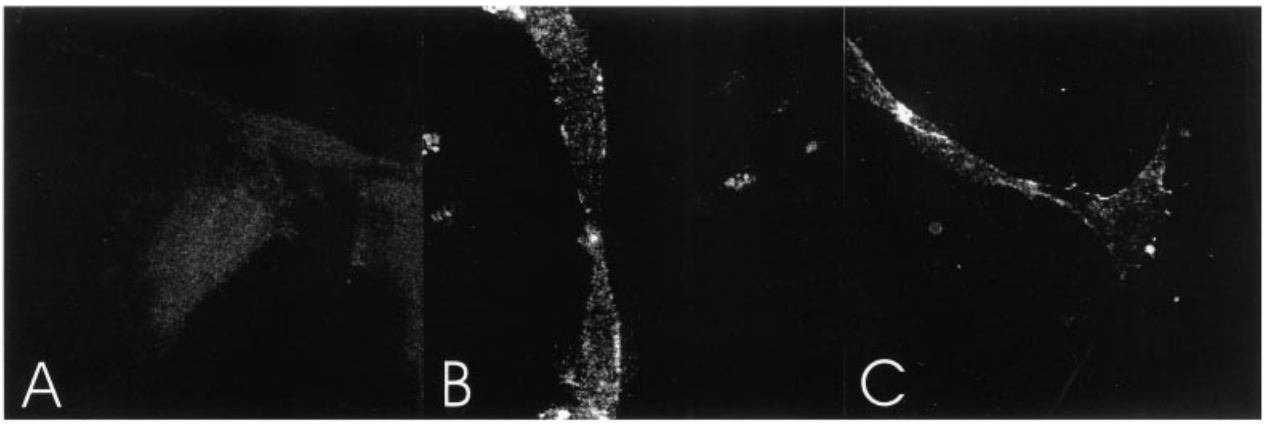

Figure 1 Effect of activated neutrophils on ECP oxidation of endothelial cells

HUVEC were cultured for 15 min at 37 °C in the presence of non-permeable TyrFluo (6 μM) under control and oxidative conditions. After removal of excess probe by washing in PBS, CLSM images were taken for (**A**) control conditions, (**B**) in the presence of PMA-activated neutrophils (2 μg/ml), (**C**) in the presence of HRP (4 munits/ml) and H_2O_2 (10 μM). The data are from one representative experiment.

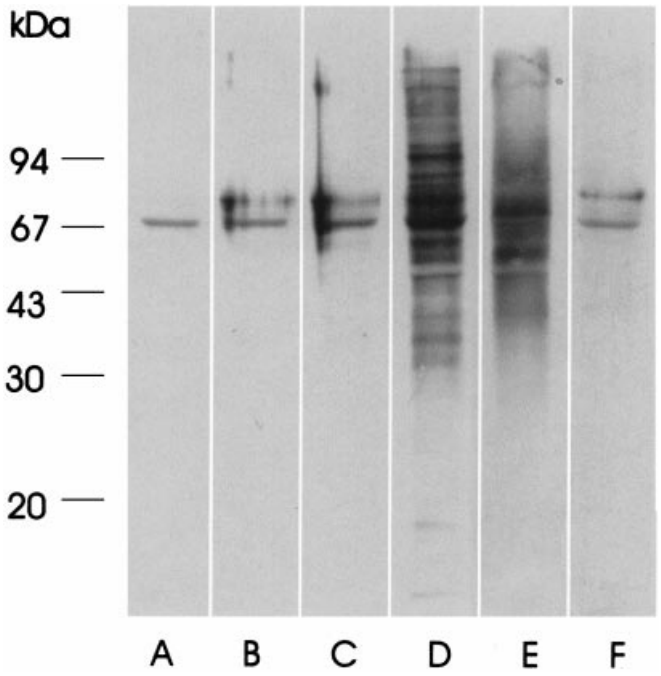

Figure 2 Pattern of ECP oxidation of endothelial cells exposed to activated neutrophils, HRP and H_2O_2

ECP oxidation was determined during 15 min of incubation at 37 °C under various conditions of oxidative stress in the presence of TyrFluo (6 μM). After SDS/PAGE, followed by Western blotting, the labelled proteins were stained with ECL® using a peroxidase-coupled antibody raised against fluorescein. (**A**) Loading control (no probe); (**B**) control conditions; (**C**) non-activated neutrophils; (**D**) PMA-activated neutrophils (2 μg/ml); (**E**) HRP (4 munits/ml) and H_2O_2 (10 μM); (**F**) H_2O_2 (100 μM). The data are from one representative experiment.

H_2O_2 (Figure 2E). In the absence of HRP, H_2O_2 (0.1 mM) did not induce ECP oxidation (Figure 2F) and gave a labelling pattern similar to the controls.

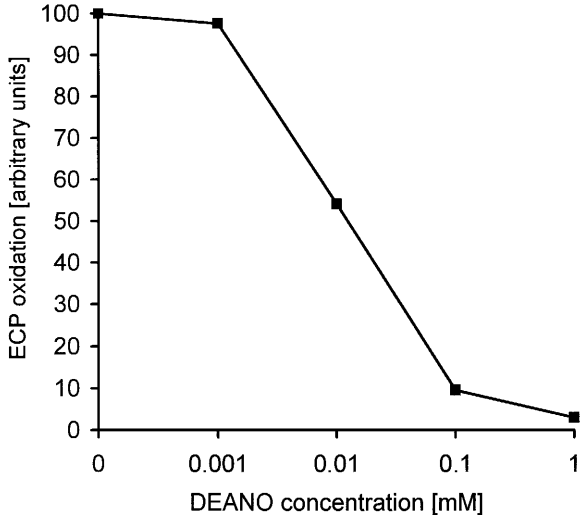

Figure 3 Effect of DEANO on ECP oxidation of endothelial cells induced by activated neutrophils

Endothelial cells were cultivated for 15 min in the presence of activated neutrophils by PMA (2 μg/ml), TyrFluo (6 μM) and different concentrations of DEANO. After the experiment, the cells were lysed, labelled proteins were collected and analysed by SDS/PAGE followed by Western blotting. The data are from one representative experiment.

Effect of DEANO

DEANO was used as a NO donor to determine its effect on ECP oxidation induced by activated neutrophils. As shown in Figure 3, DEANO (0.1–1 mM) almost completely inhibited the protein oxidation. In the absence of neutrophils, DEANO did not induce ECP oxidation in HUVEC except at 1 mM, when a low level of dityrosine formation was observed (results not shown). ECP oxidation induced by the HRP/H_2O_2 system increased with time (Figures 4A–4C). DEANO (1 mM) considerably decreased dityrosine formation at each time point of incubation (Figures 4D–4F). At a lower concentration of DEANO

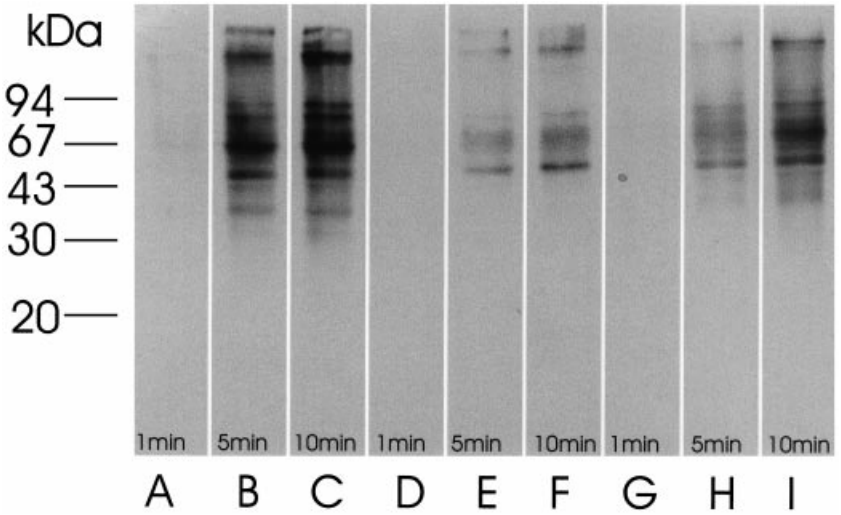

Figure 4 Time-dependent effect of DEANO on ECP oxidation of endothelial cells induced by HRP/H_2O_2

Endothelial cells were cultivated for 1, 5 and 10 min in the presence of non-permeable TyrFluo (6 μM). Oxidative stress was induced by HRP (4 munits/ml) and H_2O_2 (10 μM), DEANO was used at 0–1 mM concentrations. (**A**–**C**) The level of ECP oxidation after 1, 5 and 10 min of incubation in the presence of HRP and H_2O_2. (**D**–**F**) Effect of DEANO (1 mM) during 1, 5 and 10 min incubation. (**G**–**I**) Effect of DEANO (0.01 mM) during 1, 5 and 10 min incubation. The data are from one representative experiment.

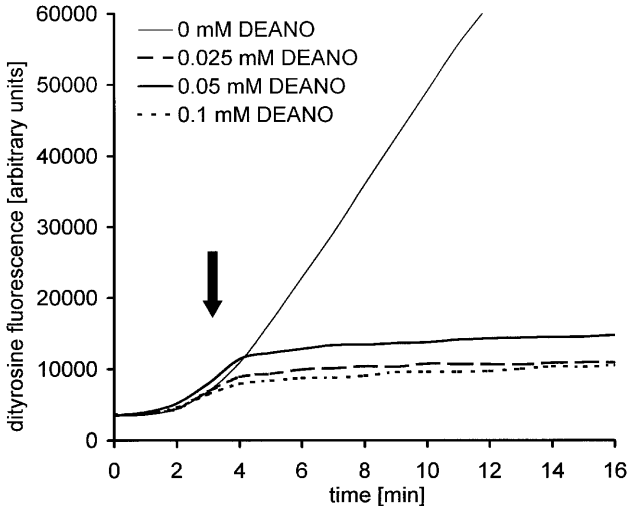

Figure 5 Concentration-dependent effect of DEANO on exogenous L-tyrosine oxidation by activated neutrophils

L-tyrosine (0.5 mM) was incubated in Hanks buffer at 37 °C with gentle mixing. Oxidative stress was induced by neutrophils (3.3×10^5 cells/ml) activated by PMA (2 μg/ml) (at the point of the arrow). Various concentrations of DEANO (0–0.1 mM) was added. During the incubation, dityrosine concentration was determined by measurement of fluorescence (λ_{Ex}, 325 nm; λ_{Em}, 405 nm). The data are from one representative experiment.

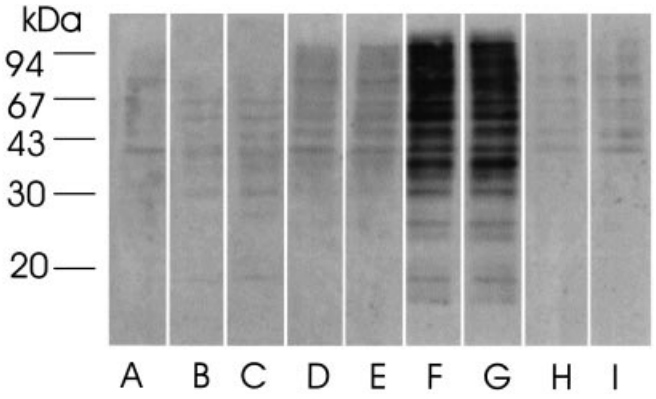

Figure 6 Pattern of ICP oxidation of endothelial cells exposed to activated neutrophils, HRP and H_2O_2

The experiments were carried out using permeant acetylTyrFluo (6 μM). After 5 min of loading, the medium was changed to remove the excess probe, and the cells were subjected to various conditions of oxidative stress for 15 min at 37 °C. After SDS/PAGE, followed by Western blotting, the labelled proteins were stained with ECL® using a peroxidase-coupled antibody raised against fluorescein. (**A**) Control conditions; (**B**) non-activated neutrophils; (**C**) PMA (2 μg/ml)-activated neutrophils; (**D**) H_2O_2 (10 μM); (**E**) HRP (4 munits/ml) and H_2O_2 (10 μM); (**F**) H_2O_2 (100 μM); (**G**) HRP (4 munits/ml) and H_2O_2 (100 μM); (**H**) H_2O_2 (100 μM) and catalase (250 units/ml) and (**I**) HRP (4 munits/ml), H_2O_2 (100 μM) and catalase (250 units/ml). The data are from one representative experiment.

(0.01 mM), the inhibition of dityrosine formation was much less evident (Figures 4G–4I).

Activated neutrophils induce exogenous L-tyrosine oxidation as detected by fluorescent dityrosine formation (Figure 5). After a lag time of 2 min, dityrosine formation increased linearly with time. Under these conditions, addition of DEANO (0.025–0.1 mM) instantaneously inhibited dityrosine formation, which indicated a very efficient inhibition of tyrosyl radical formation.

ICP oxidation in endothelial cells

Oxidation of ICP was studied by using membrane-permeable acetylTyrFluo (Figure 6). After diffusion of this non-polar probe into the HUVEC, it is converted by non-specific esterases into TyrFluo, which is retained within the cells during the time of the experiment. Under control conditions as well as in the presence of non-activated neutrophils, little oxidation was observed

(Figures 6A and 6B). Under conditions where activated neutrophils led to ECP oxidation, ICP oxidation was similar to control (Figures 6B and 6C). This strongly suggests that the effects of the oxidative burst are restricted to proteins exposed to the medium. A slightly enhanced ICP oxidation was observed when HUVEC was incubated with 4 munits HRP/10 μM H_2O_2 (Figure 6E). This level of oxidation was similar to that observed with 10 μM H_2O_2 alone (Figure 6D). ICP oxidation was clearly evident when HUVEC were incubated with 100 μM H_2O_2 (Figure 6F). Incubation with 4 munits HRP/100 μM H_2O_2 resulted in a comparable oxidation (Figure 6G). Under the latter conditions of oxidative stress, addition of catalase to the medium completely prevented ICP oxidation (Figures 6H and 6I). The basal oxidation of ICP was modulated neither by the NO synthase inhibitor N^G-nitro-L-arginine methyl ester nor by the NO donors *S*-nitroso-*N*-acetyl-DL-penicillamine and DEANO. The antioxidant Trolox also failed to have an effect (results not shown).

DISCUSSION

In the present study, we have shown that the oxidative burst generated by PMA-activated neutrophils oxidatively modify a large number of ECPs of HUVEC at the level of tyrosine residues. Under these conditions, the oxidation of ICP was not detected. These results suggest that the tyrosine residues of plasma membrane proteins could serve as a substrate for myeloperoxidase (MPO) enzymic activity. The absence of ICP oxidation after 15 min of oxidative burst suggests that the oxidizing agents released by neutrophils do not diffuse into the HUVEC during this period. This may indicate that the oxidation of ECP does not affect the membrane integrity under brief stress conditions. Previously, it was observed that neutrophil-derived oxidants did increase the permeability of endothelial cells and altered receptors located at the membrane surface [14,15]. Hence, it may be concluded that, to protect endothelial cells from membrane damage by activated neutrophils, antioxidant intervention should be performed at an early stage of the oxidative burst.

Neutrophils activated by PMA secrete MPO and O_2^-, which is then converted into H_2O_2 by superoxide dismutase [16]. The MPO/H_2O_2 system can convert tyrosine residues into tyrosyl radicals, leading to the formation of dityrosine cross-link in proteins. O_2^- present is able to scavenge for tyrosyl radicals. We have also shown that the oxidative burst generated by activated neutrophils is restricted to the oxidation of ECP of HUVEC. This agrees with MPO not being able to cross the plasma membrane. A similar pattern of ECP oxidation was observed when HRP/H_2O_2 was used as an oxidation system. HRP/H_2O_2 resulted in ICP oxidation to a level comparable with that of H_2O_2 alone. This oxidation is prevented when catalase is added to the medium, indicating that ICP oxidation is dependent on H_2O_2 diffusing into the cells. These observations are in agreement with a previous study in which H_2O_2 was shown to oxidize ICPs of rat-1 fibroblasts [12]. Fenton reaction converts H_2O_2 into the very reactive hydroxyl radical ($OH^\bullet$) resulting in an oxidative stress inside the cell. Moreover, H_2O_2 may induce intracellular Ca^{2+} oscillations in human endothelial cells [17]. Calcium can stimulate O_2^- production in mitochondria and also NO synthase(s) [18].

In the present study, we have shown that the NO donor DEANO inhibited ECP oxidation induced by both activated neutrophils and by HRP/H_2O_2. DEANO is a spontaneous NO donor with a half-life of 2 min in a phosphate buffer, pH 7.4, at 37 °C [19]. NO may inhibit the activity of MPO and HRP by interaction with the haem moiety as a part of the catalytic centre. Stable low-spin six-co-ordinate complexes of NO with MPO [i.e. MPO–Fe(III)·NO and MPO–Fe(II)·NO] have been detected [20]. It has also been shown that NO inhibits oxidation of low-density lipoproteins induced by peroxyl radicals [21]. From experiments with exogenous L-tyrosine it was found that the formation of dityrosine by activated neutrophils was inhibited by DEANO and that this inhibitory effect disappeared when the concentration decreased to 20 nM (calculated using half-life of DEANO; results not shown). Autoxidation of NO by O_2 results in the formation of NO_2^- as a primary product [22]. Nitrite can be further oxidized by haem peroxidases in the presence of H_2O_2 to NO_3^-, which is able to nitrate tyrosine [23,24]. However, in the present study this nitrosylation reaction is unlikely, since NO inhibits MPO activity.

NO can be synthesized both by endothelium and by neutrophils, but the role of an inducible form of NO synthase in some pathological conditions is controversial [25]. NO also mediates the early and late phases of ischaemic preconditioning; exogenous NO donors can reduce the size of ischaemic infarct [26]. The role of exogenous NO in preventing oxidative damage of the endothelium under different pathological conditions is not fully understood [27,28]. Our results demonstrate that oxidants produced during excessive activation of neutrophils may contribute to the oxidative modification of ECP of endothelial cells and that NO donor(s) may protect these cells from the oxidative damage.

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