



Crucial role of local peroxynitrite formation in neutrophil-induced endothelial cell activation

Hae-Young Sohn^{a,*}, Florian Krotz^b, Stefan Zahler^b, Torsten Gloe^b, Matthias Keller^a, Karl Theisen^a, Thomas M. Schiele^a, Volker Klaus^a, Ulrich Pohl^b

^aDivision of Cardiology, Medizinische Klinik Innenstadt, Ludwig-Maximilians-University Munich, Ziemssenstrasse 1, 80336 Munich, Germany

^bInstitute of Physiology, Ludwig-Maximilians-University Munich, Munich, Germany

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Abstract

Introduction and methods: The reaction of superoxide anions and NO not only results in a decreased availability of NO, but also leads to the formation of peroxynitrite, the role of which in the cardiovascular system is still discussed controversially. In cultured human endothelial cells, we studied whether there is a significant interaction between endothelial NO and neutrophil-derived superoxide anions in terms of endothelial peroxynitrite formation. We particularly studied whether a significantly higher redox-stress can be found in those endothelial cells directly adjacent to an activated neutrophil. **Results:** A considerable part of the 2,7-dihydrodichlorofluorescein signal in endothelial cells was due to oxidation by peroxynitrite. Providing superoxide radicals by enzymatic source or by the neutrophil respiratory burst increased the fluorescence, which was attenuated by blockade of endothelial NO-synthase, suggesting that peroxynitrite was formed from neutrophil- or extracellular enzyme-derived superoxide and endothelial NO. Considerably higher fluorescence intensity was observed in endothelial cells in direct neighborhood to a neutrophil. This was particularly pronounced in the presence of a NO-donor and was accompanied by a strong activation of NF- κ B and increased expression of E-selectin in these cells. **Conclusion:** Endothelial cells adjacent to neutrophils may have elevated levels of peroxynitrite that result in an increased expression of adhesion molecules. Such cells might represent a preferential site for adhesion and migration of additional neutrophils when simultaneously high concentrations of NO and neutrophil-derived superoxide are present.

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1. Introduction

The function of cardiovascular cells can be profoundly altered by affecting their redox status [1]. Vascular cell proliferation, expression of adhesion molecules, production of cytokines and activation of important transcription factors have been shown to be modulated by reactive oxygen species (ROS) [2]. Among the various ROS, superoxide anions (O_2^-) derived from endothelial cells play an important role as they rapidly inactivate endothelial-derived nitric oxide (NO), resulting in an impairment of NO-dependent vasoprotective effects [1].

Within the vessel wall, endothelial cells seem to represent the main cellular source of O_2^- [3,4]. Endothelial cells display a constitutive release of O_2^- in the nanomolar range which stems from a NAD(P)H-dependent oxidase. This enzyme seems to represent an isoform of the neutrophil-type NADPH oxidase including the subunits gp91^{phox} as the site of electron transfer [5–7]. In vessels isolated from hypertensive or hyperlipidemic animals, the activity of this enzyme is found to be increased, which supports the view that chronically enhanced vascular O_2^- -production is crucially involved in the development of hypertension and the pathogenesis of atherosclerosis [1].

O_2^- rapidly reacts with NO which not only results in decreased availability of NO, but also leads to the forma-

*Corresponding author. Tel.: +49-89-5160-2266; fax: +49-89-5160-2410.

E-mail address: sohn@lrz.uni-muenchen.de (H.-Y. Sohn).

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tion of peroxynitrite (ONOO^-), the role of which in the cardiovascular system is still discussed controversially [8–10]. ONOO^- , in micro- and millimolar concentrations, has been shown to have direct cytotoxic effects [11,12]. Very recently, van der Loo et al. demonstrated that an enhanced ONOO^- -formation resulting from increased endothelial O_2^- -production is associated with vascular aging [13]. In contrast, ONOO^- at lower concentrations has been suggested to promote vasoprotective effects. In particular, a cardioprotective role of ONOO^- in myocardial ischemia/reperfusion (I/R)-injury has been reported [14]. However, studies dealing with effects of ONOO^- in vivo are rare and in many in vitro studies, the concentrations of ONOO^- used are very high and presumably non-physiological.

During activation of polymorphonuclear leukocytes (PMN), such as in acute cardiovascular events, a burst-like production of high amounts of O_2^- occurs which is thought to increase the formation of ONOO^- as well by reaction with surrounding NO [15]. In such situation, NO may be a limiting factor. It is, however, unclear how much NO really is available and what its source may be. Fukuyama et al. reported that nitration of proteins induced by activated PMN was eliminated when PMN had been pretreated with an inhibitor of NO-synthase. This would indicate that ONOO^- is generated already in PMN and thereafter released to the environment [16,17]. Alternatively, or perhaps in addition, PMN can interact directly with single endothelial cells, making NO of these endothelial cells available for local ONOO^- -formation with O_2^- of the PMN. Exogenously given NO-donors in acute coronary syndromes provide an additional condition for high ONOO^- accumulation in those endothelial cells directly exposed to an attached PMN generating O_2^- . Therefore, at a very local level, there may be drastically high concentrations of ONOO^- that could alter cell function and integrity. This local effect has never been closely examined.

Very recently, Matata et al. reported that ONOO^- in micromolar concentrations can activate the transcription factor nuclear factor kappa B (NF- κ B) subunit p65 [18], known to control the expression of endothelial adhesion molecules [1,19]. These results suggest that formation of high intracellular ONOO^- concentrations, particularly in endothelial cells directly adjacent to PMN, should increase the expression of adhesion molecules and thus, possibly facilitate further contacts of PMN with this same endothelial cell.

In this study, we examined whether there is significant interaction between endothelial NO and PMN-derived O_2^- in terms of endothelial ONOO^- formation, especially in those single endothelial cells which are in direct contact with an activated PMN. Also, it has been tested whether inhibition of the local ONOO^- formation by blockade of endothelial NO-synthase influences respiratory burst-induced activation of the NF- κ B and subsequent expression of endothelial adhesion molecules such as ICAM-1 or

E-selectin. We further studied whether exogenous NO, derived from NO-donors, can affect endothelial ONOO^- generation. Since acute cardiovascular disorders such as ischemia/reperfusion(I/R)-injury go along with respiratory burst-mediated cell damage, insights into the role of ONOO^- —generated from endogenous endothelial NO and/or by exogenous NO-donors—on endothelial cell activation are of significant clinical interest.

2. Methods

2.1. Cell culture

Endothelial cells were isolated from human umbilical veins (HUVEC) as described before [20]. The use of HUVEC was approved by the ethics committee. The cells were maintained in endothelial growth medium (Promocell, Germany) and used after confluence was reached. All cells were used in subpassage I–II.

2.2. Isolation of polymorphonuclear neutrophils (PMN)

PMN were isolated from venous blood samples of healthy volunteers as we described previously [21]. Informed consent to blood sampling and use of leukocytes was obtained from all subjects. Isolated PMN were activated by phorbol-12-myristate-13-acetate (TPA) (1 μM) and thereafter washed twice with phosphate-buffered saline to remove TPA from the PMN-suspension.

2.3. L-012 chemiluminescence

The formation of O_2^- by PMN or HUVEC induced by TPA was determined by the assay employing 8-amino-5-chloro-7-phenylpyridol[3,4-*d*]pyridazine-1,4(2H,3H) dione (L-012) [22]. Suspensions of PMN or HUVEC were incubated in modified Tyrode's buffer (135 mmol/l NaCl, 2.7 mmol/l KCl, 1.8 mmol/l CaCl_2 , 0.49 mmol/l MgCl_2 , 0.28 mmol/l M NaH_2PO_4 , 5.5 mmol/l M glucose, 20 mmol/l Hepes) containing 0.1 mmol/l L-012 and the chemiluminescence was measured using a luminometer (Berthold Lumat 9107).

2.4. 2,7-Dihydrodichlorofluoresceine fluorescence (DCFH-FL) assay

HUVEC were incubated with the membrane-permeable dye DCFH-DA (10 $\mu\text{mol/l}$) in modified Tyrode's buffer for 15 min and the fluorescence intensities (excitation 488 nm, emission >515 nm) were measured using a confocal laser scanning microscope (LSM 410, Zeiss, Germany). Values obtained are expressed as arbitrary fluorescence units (FU).

2.5. Detection of local ONOO⁻ formation

HUVEC grown on 24-well cell culture plates were pre-treated with tumor necrosis factor alpha (TNF- α , 2.5 ng/ml) for 4 h. They were then incubated with 2.5×10^5 PMN/ml and centrifuged ($500 \times g/5$ min) to facilitate adhesion of PMN to the endothelial monolayer. After 30 min, non-adherent PMN were removed by washing and the medium changed to a modified Tyrode's buffer solution containing 10 μ mol/l DCFH-DA. To detect local production of ONOO⁻ in single cells, regions of interest (ROI) were randomly placed onto single endothelial cells with or without adherent PMN and changes in fluorescence intensities were recorded with a confocal microscope (see also Fig. 3A).

2.6. Nuclear translocation of NF- κ B subunit p65

Activation of NF- κ B was determined by assessing the distribution of its subunit p65 between cytoplasm and the nucleus of HUVEC in immunofluorescence images as described before [23]. Briefly, HUVEC were fixed with buffered formaldehyde (3%) and subsequently permeabilized by submersion in 0.2% Triton X-100. The samples were then incubated with the primary antibody against p65 and finally treated with the secondary antibody linked to FITC. Fluorescence intensities were detected using a confocal microscope and the cellular distribution of p65 was measured as the ratio of its fluorescence in nucleus/cytoplasm. TNF- α was used as a positive control.

2.7. Expression of ICAM-1 and E-selectin

Overall expression levels of ICAM-1 and E-selectin in HUVEC were quantified by flow cytometry. HUVEC were detached from the culture dish and fixed with 10% CellFix (w/v) (Becton Dickinson, Germany). Resuspended cells were labeled with the respective antibodies, washed, and fluorescence measured on a FACScan flow cytometer (Becton Dickinson). The antibodies MCA675PE and MCA883F were used to detect ICAM-1 and E-selectin, respectively.

2.8. Detection of local expression of ICAM-1 and E-selectin

Confluent HUVEC grown on 24-well cell culture plates were incubated with 1×10^5 PMN/ml. Before cocubation, PMN were dye-loaded with PKH26 (Sigma, Germany) and pre-activated with TPA (1 μ mol/l). Cell plates were centrifuged ($500 \times g/5$ min) to facilitate adhesion of PMN to the endothelial monolayer. Non adherent PMN were removed by a washing procedure. After incubation for 3 h, HUVEC and adherent PMN were fixed with buffered formaldehyde (3%). In pilot experiments, incubations for longer than 3 h had resulted in significant

detachment of PMN from the endothelial cell layer. The samples were incubated with the primary mouse-antibody against human ICAM-1 and E-selectin and subsequently treated with the secondary antibody linked to Alexa Fluor 546 (Molecular Probes). Local fluorescence intensities were studied and quantified using a confocal microscope as described above.

2.9. Nitrotyrosine staining

HUVEC were fixed with buffered formaldehyde (3%) and permeabilized using 0.2% Triton X-100. The samples were then incubated with the primary antibody against nitrated proteins and treated with the secondary antibody linked to FITC. Fluorescence intensities were then assessed using a confocal microscope as described above.

2.10. Materials and chemicals

MCA675PE and MCA883F were purchased from Serotec (Kidlington, UK). DCFH-DA was from Molecular Probes (USA). L-012 was provided by Dr K. Schoenafinger (Aventis, Frankfurt, Germany). The antibodies against the subunit p65 and nitrotyrosine were obtained from Santa Cruz (USA). All other drugs were obtained from Sigma (Deisenhofen, Germany).

2.11. Statistical analysis

Statistical comparisons of paired experiments were carried out using the Wilcoxon signed rank test. Differences were considered significant at an error probability level of $P < 0.05$. All results are expressed as means \pm S.E.M.

3. Results

3.1. Effect of protein kinase C activation on O₂⁻ formation of PMN and endothelial cells

HUVEC exhibited a low basal O₂⁻-production which was increased approximately 4.5-fold by TPA (1 μ mol/l) within 10 min. Suspensions of isolated human PMN showed a stronger basal release of O₂⁻ which was increased after stimulation with the same concentration of TPA by \sim 600-fold. Activated PMN therefore produced >2500 -fold more O₂⁻ per cell than stimulated endothelial cells in the L-012 chemiluminescence assay (1.4 vs. 3518 relative light units ($\times 1000$)/ 10^6 cells, $n=4$).

3.2. Effect of exogenous addition of O₂⁻ on endothelial ROS-formation

HUVEC were loaded with the ROS-sensitive fluorescent dye DCFH-DA and the effects of exogenous application of

O_2^- were tested using either the O_2^- generating system xanthine (0.1 mmol/l)/xanthine oxidase (5 mU/ml, XO) or by adding TPA-preactivated PMN to the endothelial cell supernatant. X/XO increased the DCF-FL intensity in HUVEC 3.8-fold within 10 min (Fig. 1A, $n=4$). This was nearly abolished in the presence of SOD (250 U/ml, $P<0.01$, results not shown). Pre-incubation of HUVEC with the NO-synthase blocker L-NA (30 μ mol/l) also markedly attenuated the X/XO-induced increase in DCF-FL by 66.3% ($n=4$, $^{\#}P<0.01$ vs. X/XO) indicating a high sensitivity of DCFH for ONOO $^-$. Accordingly, simultaneous treatment with the X/XO-system and the NO-donor SNAP (30 μ mol/l) further enhanced the DCF-signal (7.3-fold; Fig. 1, $n=4$, $^{\#}P<0.01$ vs. X/XO). Addition of SNAP alone slightly increased DCF-FL ($P=0.051$, $n=7$). Immunofluorescence detection of nitrated proteins revealed similar results (Fig. 1B, $n=3$).

O_2^- derived from suspensions of TPA-stimulated PMN (2.5×10^5 /ml) also induced a marked increase in DCF-FL in HUVEC (>5-fold, Fig. 2A,B). The latter effect was nearly abolished in the presence of SOD (250 U/ml, Fig. 2A,B). Direct incubation of HUVEC with this high concentration of TPA (1 μ mol/l) increased the DCF-FL by only 58.2% (Fig. 2A, $n=8$, $P<0.05$). Pre-incubation of only the endothelial cells with the PKC-blocker chelerythrine (30 μ mol/l) did not prevent the PMN-in-

duced increase in DCF-FL (2.5 ± 0.2 vs. 2.7 ± 0.2 increase in FU/min, $n=8$, n.s.). Inhibition of neutrophil NO-synthase by L-NA (30 μ mol/l) prior stimulation with TPA did not significantly affect the DCF-FL in endothelial cells indicating no direct peroxynitrite formation by PMNs (2.5 ± 0.2 vs. 2.2 ± 0.2 increase in FU/min, $n=8$, $P=0.08$).

3.3. Effect of PMN-derived O_2^- on local endothelial ROS-formation

In order to study whether PMN-derived O_2^- induces a higher increase in ROS formation in those endothelial cells which are in direct contact with an activated PMN, 'regions of interest' were randomly placed onto single endothelial cells and the change in DCF-FL intensities were studied within the same cell culture plate in cells with and without adherent PMN (ratio HUVEC/PMN= \sim 20–30:1). A typical pattern including ROI (squares) is shown in Fig. 3A. HUVEC with attached PMN exhibited a markedly stronger DCF-FL than endothelial cells without adjacent PMN (Fig. 3B, $n=18$, $P<0.01$). SOD (250 U/ml) as well as the NO-synthase blocker L-NA (30 μ mol/l) attenuated this local increase in DCF-FL by 90.1 and 45.5%, respectively (Fig. 3C, $n=6$, $P<0.01$ and $P<0.05$, respectively). Similar results were obtained using the calcium ionophore A23187 (1 μ mol/l) as stimulus for

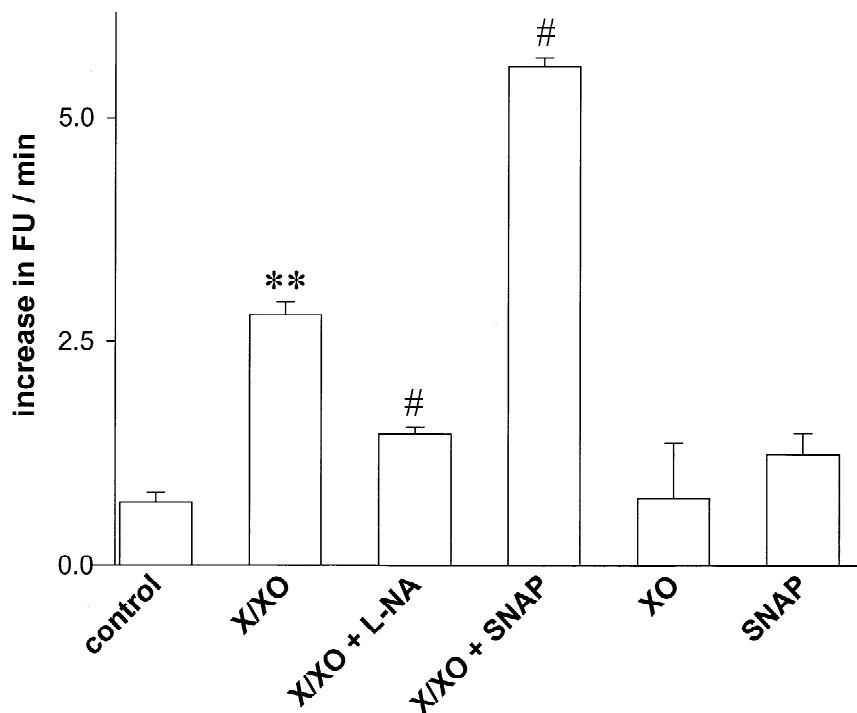


Fig. 1. Effect of reactive oxygen species on DCF fluorescence in HUVEC. The O_2^- -generating system X/XO (0.1 mmol/l per 5 mU/ml) increased DCF-FL 3.8-fold within 10 min ($n=4$, $^{**}P<0.01$ vs. X/XO). Pre-incubation of HUVEC with the NO-synthase blocker L-NA (30 μ mol/l) significantly attenuated the X/XO-induced increase in DCF-FL ($n=8$, $^{\#}P<0.01$ vs. X/XO), as did SOD (250 U/ml, not shown). Simultaneous addition of X/XO and the NO-donor SNAP (30 μ mol/l) markedly enhanced DCF-FL ($n=8$, $^{\#}P<0.01$ vs. X/XO). XO alone did not affect the DCF-signal while SNAP increased it ($n=7$, $P=0.051$). (B) Increase in peroxynitrite generation was assessed using antibody against nitrated proteins. L-NA (30 μ mol/l) nearly abolished the X/XO-induced increase in nitrotyrosine staining while SNAP (30 μ mol/l) considerably augmented it ($n=3$).

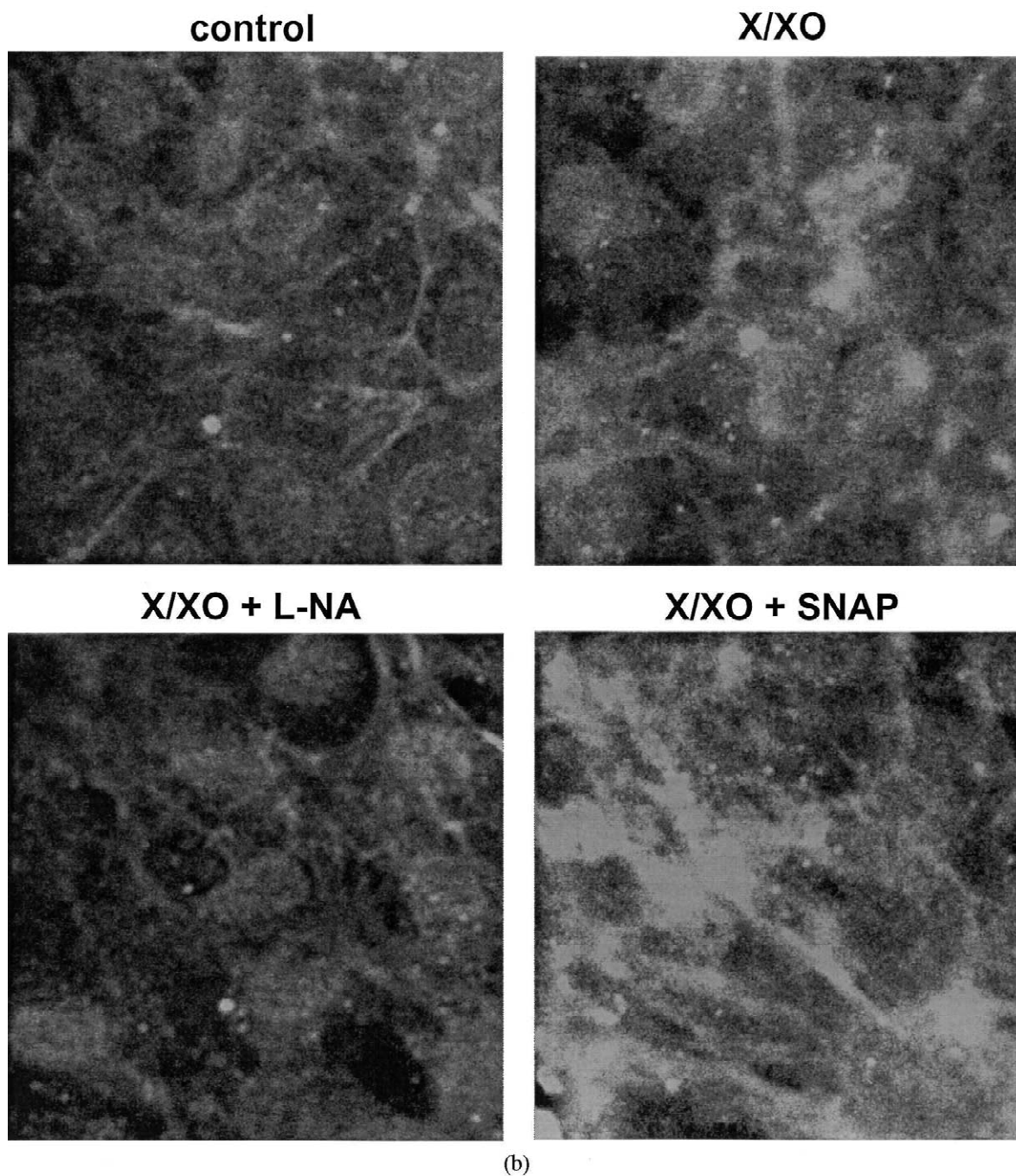


Fig. 1. (continued)

PMN (data not shown). When quiescent PMN were used, no differences in DCF-FL could be observed (0.98 ± 0.34 vs. 0.93 ± 0.23 FU/min, $n=12$, n.s.).

3.4. Effect of PMN-derived ROS on endothelial ICAM-1 and E-selectin expression

As shown in Fig. 4A,B, addition of a suspension of TPA-preactivated PMN (10^6 /ml) to the endothelial layer increased ICAM-1 and E-selectin expression in endothelial

cells after 6 h by 70.4 and 51.4%, respectively ($n=5$, $*P<0.05$ vs. control). Inhibition of endogenous NO (L-NA, $30 \mu\text{mol/l}$) was without effect (data not shown). The NO-donor SNAP at a concentration of $3 \mu\text{mol/l}$ was not effective but higher concentrations of 30 and $100 \mu\text{mol/l}$ (data not shown), further augmented PMN-induced expression of ICAM-1 and E-selectin (Fig. 4A,B, $^{\#}P<0.05$ vs. PMN alone). SNAP alone had no effect. SOD (250 U/ml) as well as scavenging NO by hemoglobin ($10 \mu\text{mol/l}$, $n=7$, data not shown) significantly attenuated the effects of

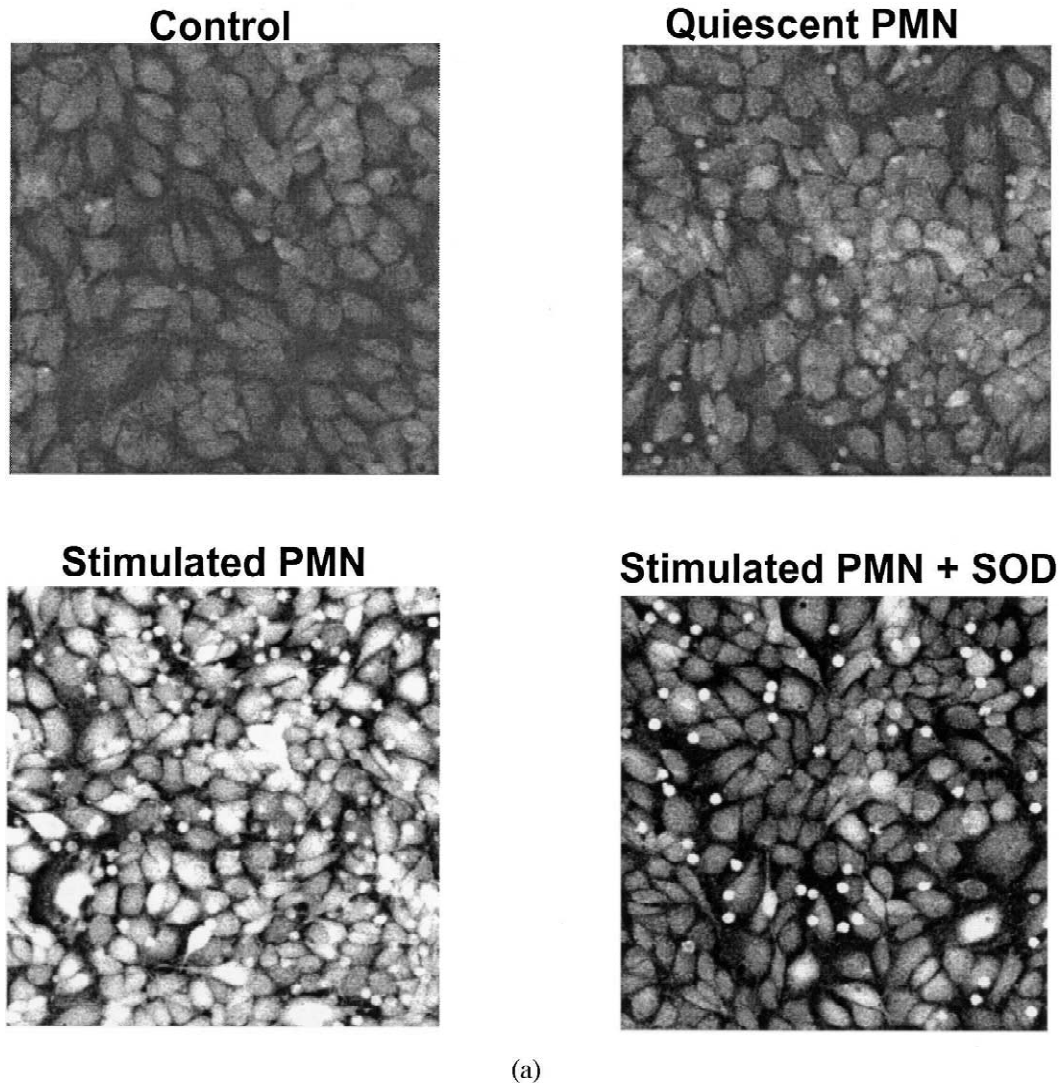


Fig. 2. Effect of PMN-derived O_2^- on DCF-FL in HUVEC. (A,B) Suspension of quiescent PMN did not significantly affect DCF-FL while pre-activation of PMN with TPA (1 $\mu\text{mol/l}$) strongly enhanced the DCF-signal in endothelial cells. This effect was nearly abolished in the presence of SOD (250 U/ml). Compared to the effects of activated PMN, direct incubation of HUVEC with TPA (1 $\mu\text{mol/l}$) resulted in a small increase in DCF-signal (left).

SNAP. In these experiments, TNF- α was exclusively used as a positive control to induce expression of endothelial adhesion molecules (Fig. 4A,B).

Fig. 5A,B demonstrates that HUVEC adjacent to PMN (arrows) exhibited a markedly stronger local E-selectin expression in the presence of SNAP (30 $\mu\text{mol/l}$) than cells without contact to PMN. There were, however, no apparent differences between HUVEC adjacent to PMN and control HUVEC concerning local expression of ICAM-1 (data not shown).

3.5. Effect on NF- κ B activation in endothelial cells

In immunofluorescence assays, exposure of HUVEC to exogenous O_2^- (X/XO; 300 $\mu\text{mol/l}$ per 10 mU/ml) significantly enhanced p65-translocation into the nucleus (Fig. 6), an action which was not influenced by L-NA (30

$\mu\text{mol/l}$, $n=5$), or catalase (1000 U/ml, data not shown) when given individually. SOD (250 U/ml) decreased the translocation of p65 into the nucleus. Simultaneous addition of the NO-donor SNAP (30 $\mu\text{mol/l}$) and X/XO to HUVEC, resulted in a stronger translocation of p65 into the nucleus than O_2^- alone (Fig. 6, $n=5$). The ratio of the cellular distribution of p65 (nucleus/cytoplasm) is shown in Table 1. Treatment of HUVEC with TNF- α also markedly increased p65-translocation and served as a positive control for our experiments (Fig. 6).

4. Discussion

The present study demonstrates that the production of O_2^- of adherent PMN selectively induces high oxidative stress in those endothelial cells directly adjacent to the

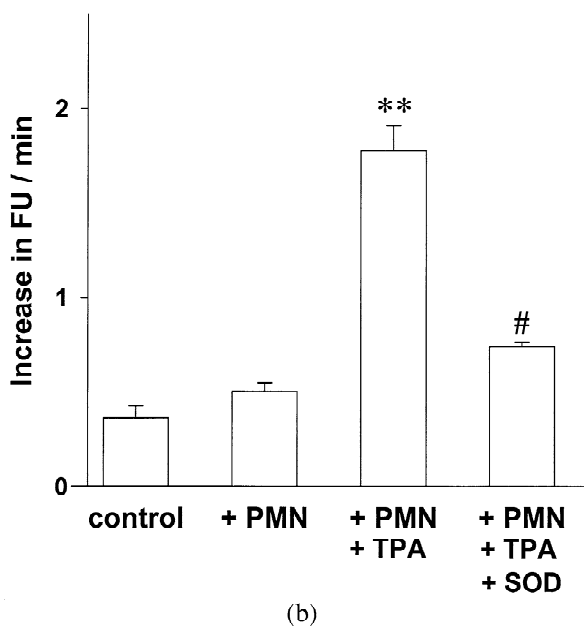


Fig. 2. (continued)

PMN. The consequences were enhanced expression of adhesion molecules and translocation of NF- κ B subunits into the cell nucleus. Blockade of endothelial NO-production by the NOS-inhibitor L-NA significantly attenuated this stress. Accordingly, the NO-donor SNAP not only further increased DCF-FL but also resulted in a stronger increase in endothelial ICAM-1/E-selectin expression and activation of the redox-sensitive transcription factor NF- κ B.

2,7-Dichlorodihydrofluorescein, commonly known as dichlorofluorescein, is not specific for one single radical but rather detects various reactive nitrogen and oxygen species in cells [24]. In our study, simultaneous exogenous addition of both NO and O_2^- , was followed by an approximately twofold stronger increase of DCF-FL in HUVEC compared to treatment with O_2^- alone, an effect which was inhibitable by SOD. These results suggest that a considerable part of the DCF-FL signal could indeed be attributed to the formation of ONOO $^-$ within endothelial cells. Immunofluorescence assessment of nitrated proteins supported these finding showing comparable results. A significant role for H_2O_2 can be ruled out, since SOD is known to dismutate O_2^- to H_2O_2 and yet as SOD abolished the X/XO-induced DCF-FL. Accordingly, PMN-induced oxidation of DCFH within endothelial cells was significantly attenuated when endothelial cells had been selectively pretreated with a NOS-inhibitor, indicating that endothelium-derived NO interacts with PMN-derived O_2^- to form ONOO $^-$. This is in accordance with recent findings that DCFH, when used in living cells, is efficiently oxidized by ONOO $^-$ [25]. Therefore, under conditions where NO and O_2^- are produced simultaneously, oxidation of DCFH is likely to predominantly include a ONOO $^-$

component [25,26]. In agreement with this, Crow et al. have recently shown in systematic in vitro studies that neither H_2O_2 nor O_2^- produce significant DCFH-oxidation, while SIN-1, known to produce NO and O_2^- simultaneously, induced DCFH-oxidation [27]. Thus, DCFH-oxidation in endothelial cells may involve more than one oxidant but inhibition of NO-synthase might be, at present, the most straightforward approach to determine whether ONOO $^-$ is involved. In the presence of L-NA, DCFH can be oxidized by O_2^- and its related radicals.

Endothelial but not PMN-derived NO [15] seems to be the main reaction partner in forming ONOO $^-$. Su et al. recently suggested that PMN-induced endothelial cell injury is mediated by formation of O_2^- but not by ONOO $^-$, since the authors failed to detect nitrotyrosine residues in endothelial cell proteins [28]. This apparent difference might be due to the higher sensitivity of DCFH towards ONOO $^-$. DCFH is oxidized by ONOO $^-$ already at concentration levels that do not yet affect tyrosine nitration. Moreover, selective pre-incubation of only the PMN with L-NA prior to activation with TPA did not significantly affect the DCFH-oxidation in endothelial cells.

In the present study, we show that activated PMN produce substantially (>2500-fold) more O_2^- per single cell than endothelial cells. Such activation occurs in I/R-injury and/or inflammation-related events within the vessel wall. Appropriately, exogenously applied NO in this situation even increases the oxidative stress by enhancing ONOO $^-$ formation to an amount that has functional effects. Our study also indicates that this accentuation occurs only in endothelial cells directly adjacent to a PMN. Such an enhanced oxidative stress, most likely caused by ONOO $^-$, presumably counteracts protective effects of NO in those cells. Recently, Gunnett et al. reported that NO-dependent relaxation is impaired in arteries after gene transfer of inducible NOS (iNOS) in vitro and in vivo, indicating that high vascular NO-formation can impair endothelial function [29].

Our study cannot provide precise information concerning the concentration of endogenous or exogenously given NO above which further activation of PMN-endothelial interaction is induced. Inhibition of endogenous NO production, however, failed to have detectable effects, suggesting that resting NO levels are not sufficient for a high enough formation of ONOO $^-$. Only further addition of the exogenous NO-donor SNAP increased NF- κ B activation and expression of adhesion molecules. This indicates that ONOO $^-$ might function as an inducer of NF- κ B and subsequent activation of adhesion molecules in high concentrations only. Since O_2^- alone is also able to induce these effects, particularly high amounts of ONOO $^-$ may be necessary to detect additional effects.

Conditions of strongly enhanced generation of both O_2^- and NO, in fact, seem to be associated with a predominant role of ONOO $^-$ for different cellular actions. Gagnon et al. reported that PMN stimulated by lipopolysaccharide (LPS)

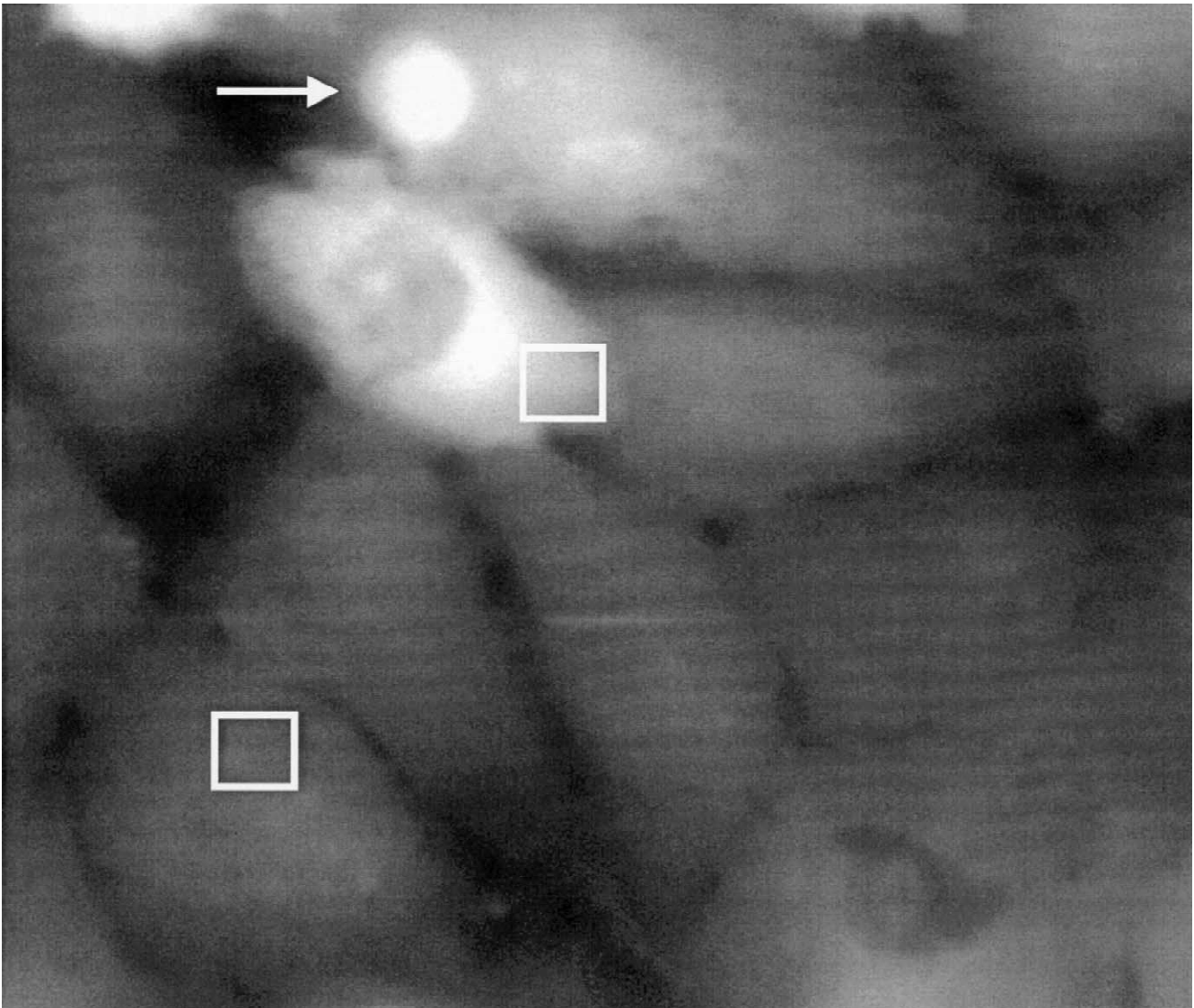


Fig. 3. Effect of direct PMN-endothelial interaction on DCF-FL. (A) Regions of interest (ROI) were placed onto single endothelial cells and fluorescence intensities were recorded over time. A typical pattern is shown including ROI for quantification of fluorescence intensities (squares). (B) HUVEC which were directly adjacent to an activated PMN showed a marked increase in DCF-FL versus HUVEC without an attached PMN ($n=18$, $P<0.01$). (C) On the average, endothelial cells adjacent to PMN generated about three times more DCF-FL (U/min) than control HUVEC. This difference in DCF-FL was attenuated by SOD (250 U/ml, $P<0.01$ vs. PMN+TPA) as well as by the NO-synthase blocker L-NA (30 $\mu\text{mol/l}$, $P<0.05$ vs. PMN+TPA).

produce high amounts of ONOO^- by activation of the inducible NO-synthase [30]. In macrophages, generation of high concentrations of NO induced by LPS and IFN- γ caused an activation of prostaglandin synthesis only in the presence of O_2^- [31], indicating that formation of ONOO^- was required. Using confocal microscopy and measurement of DCF-FL in single cells, we demonstrate here that such high concentrations of ONOO^- can occur locally in those endothelial cells which are directly adjacent to an activated PMN. In the presence of high concentrations of exogenously given NO, the latter cells significantly overexpressed E-selectin. Although we were not able to identify activation of NF- κB in single cells

using a p65-immunofluorescence assay, it is speculated that the transcription factor is involved in ONOO^- mediated E-selectin expression, since further addition of NO-donor induced a stronger p65 translocation than O_2^- alone. On the other hand, we could not find an increase in local expression of ICAM-1. In retrospect, this might be simply due to the more delayed expression of ICAM-1, since the incubation period was limited to 3 h. As described above, a main part of initially adherent PMN were detached after 3 h. The onset of E-selectin expression in HUVEC (maximum after 2–4 h) occurs sooner than that of ICAM-1 (maximum after >6 h) [32] so that no change in ICAM-1 expression could be detected after 3 h while suspensions of

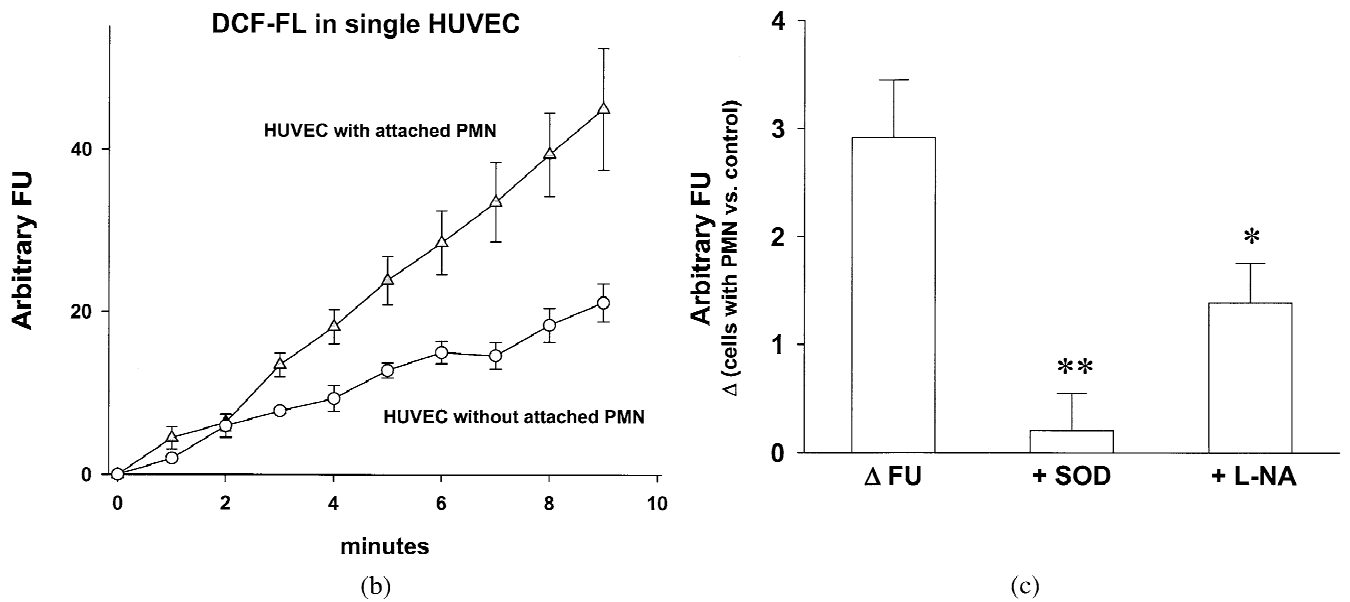


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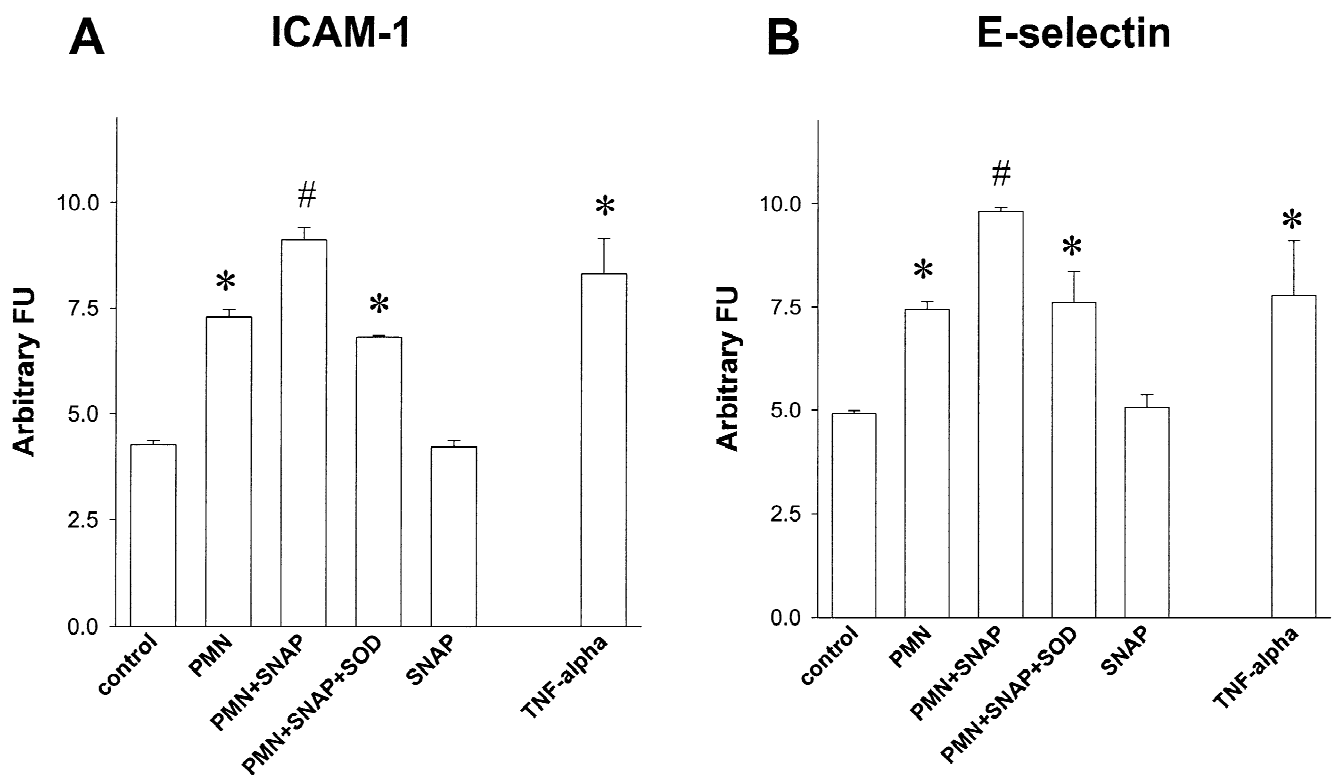


Fig. 4. Effect on endothelial ICAM-1/E-selectin expression. Expression levels of the adhesion molecules ICAM-1 and E-selectin on HUVEC were quantified by flow cytometry. Addition of activated PMN (10^6 /ml) to HUVEC increased ICAM-1 (A) and E-selectin (B) expression ($n=5$, $*P<0.05$ vs. control). In the presence of the NO-donor SNAP ($30 \mu\text{mol/l}$), PMN further increased expression of ICAM-1 and E-selectin ($n=5$, $\#P<0.05$ vs. PMN), an effect inhibited by SOD. Inhibition of endogenous NO by L-NA ($30 \mu\text{mol/l}$) did not affect PMN-induced effects (not shown). Endogenous endothelial O_2^- did not suffice to give increased expression of adhesion molecules when SNAP was added without PMN. Activation induced by TNF- α served as a positive control.

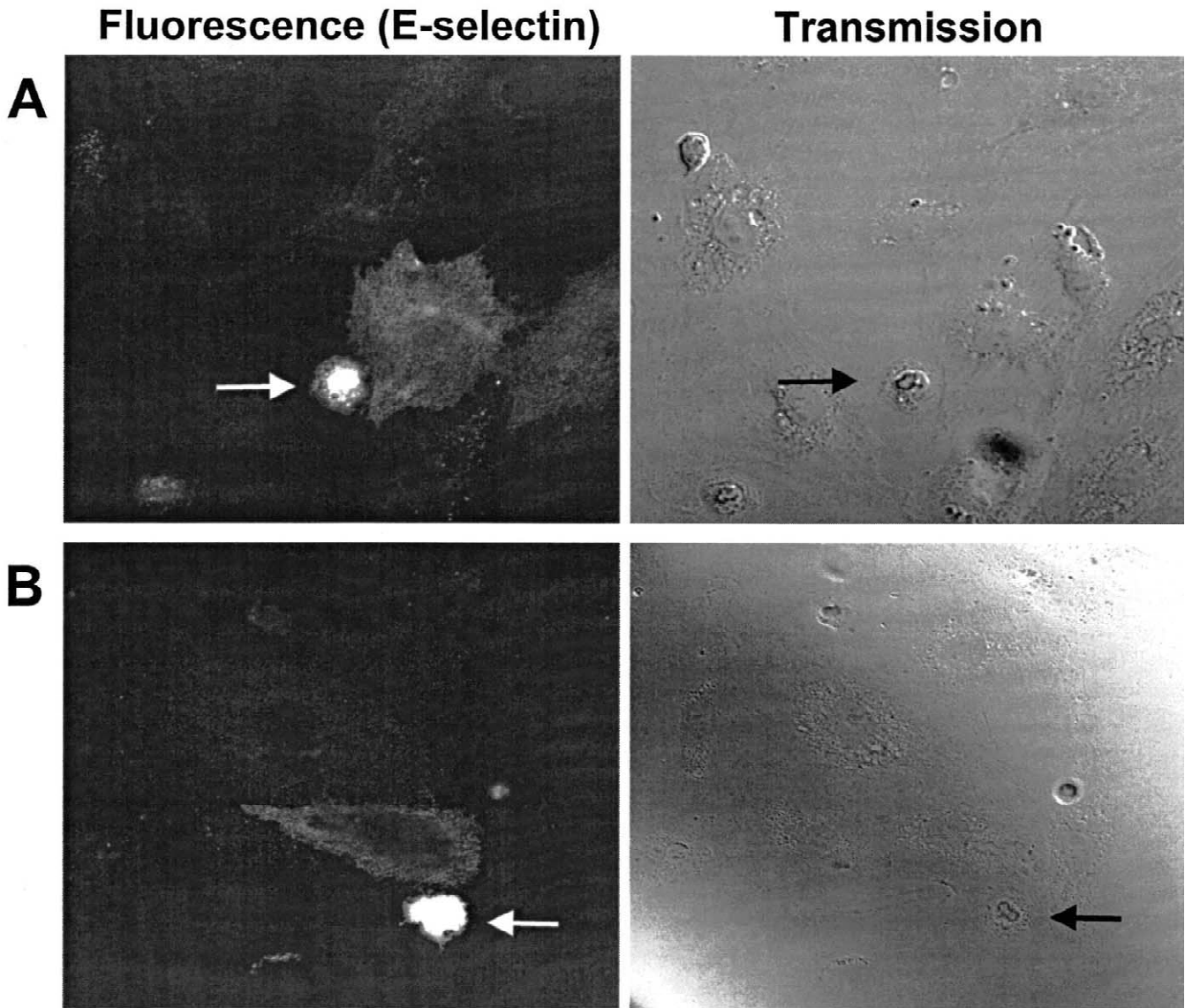


Fig. 5. Effect of direct PMN-endothelial interaction on local E-selectin expression. Local expression of E-selectin (A and B) was detected by immunofluorescence using confocal microscopy as described in Methods. Before treatment, PMN were dye-loaded to identify attached PMN (arrows, staining with PKH26). HUVEC which were in direct contact with an activated PMN exhibited a strong expression of E-selectin (secondary antibody linked to FITC) in the presence of SNAP (30 $\mu\text{mol/l}$).

PMN enable a longer incubation period (6 h). Pertinently, in these assays, an increased expression of both adhesion molecules could be detected.

In the present study, we provide evidence that PMN-derived O_2^- significantly interact with endothelial NO to form detectable amounts of ONOO^- within endothelial cells. There was, however, no evidence for a functional consequence of this level of endothelial ONOO^- production in terms of general expression of endothelial adhesion molecules. However, in endothelial cells directly neighboring an activated PMN, it was possible to demonstrate that considerably higher amounts of ONOO^- are generated, particularly in the presence of an exogenous NO-donor. The latter condition is associated with a

stronger translocation of the transcription factor NF- κB subunit p65 into the nucleus and enhanced local expression of the adhesion molecule E-selectin. We conclude that, in the cardiovascular system, a deleterious concentration of ONOO^- might arise in endothelial cells directly exposed to PMN when a simultaneous increase of both O_2^- and NO occurs. These particular endothelial cells might represent a preferred site for adhesion of additional PMN, promoting their migration into the vascular wall and subsequent cellular damage. Hence, in cardiovascular events such as I/R-injury, associated with a large number of activated PMN, the initiation of the firm neutrophil-endothelial interaction might be aggravated by oral or intravenous administration of NO-donors.

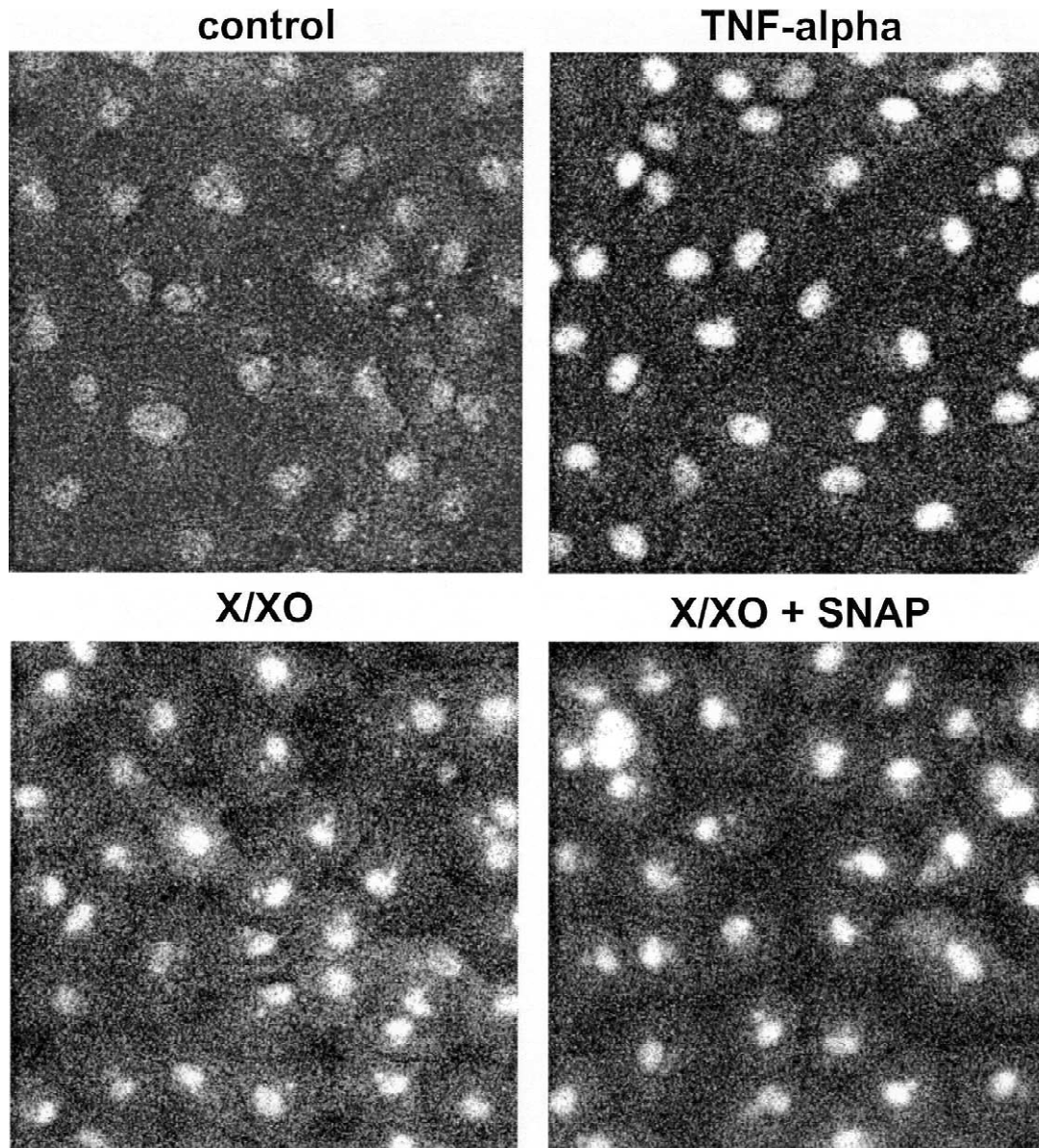


Fig. 6. Nuclear translocation of the NF- κ B subunit p65. Activation of NF- κ B in HUVEC was assessed by immunofluorescence detection of translocation of its subunit p65 into the nucleus. Treatment of HUVEC with TNF- α (2.5 ng/ml) strongly increased nuclear subunit p65 ($n=3$). Exogenously given O_2^- (X/XO; 0.3 mmol/l per 10 mU/ml) induced translocation of the subunit p65 into the nucleus ($n=5$). Simultaneous addition of SNAP (30 μ mol/l) and X/XO, enhanced nuclear translocation of p65 more than X/XO alone ($n=5$).

Table 1
Cellular distribution of the NF- κ B subunit p65 (ratio nucleus/cytoplasm);
 $n=5$

		<i>P</i> -value versus control	<i>P</i> -value versus X/XO
Control	1.34 \pm 0.04		
X/XO	1.98 \pm 0.11	<0.01	
X/XO+SNAP	2.98 \pm 0.11	<0.01	<0.01
X/XO+L-NA	1.79 \pm 0.09	<0.05	n.s.
X/XO+SOD	1.55 \pm 0.06	<0.05	<0.05
TNF- α	3.84 \pm 0.17	<0.01	<0.01

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