Peroxynitrite is not a major mediator of endothelial cell injury by activated neutrophils in vitro

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Abstract

Objective: Human polymorphonuclear leukocytes (PMN) produce nitric oxide (NO), superoxide (O\textsuperscript{2-}) and peroxynitrite (ONOO\textsuperscript{-}) upon stimulation. We investigated the role of ONOO\textsuperscript{-} in PMN-induced injury to cultured bovine aortic endothelial cells (BAEC).

Methods: BAEC were cocultured with phorbol 12-myristate 13-acetate (PMA)-activated human PMN (effector-to-target ratio, 10:1) and injury to BAEC was evaluated at intervals by \textsuperscript{51}Cr release assay. The levels of NO, O\textsuperscript{2-}, ONOO\textsuperscript{-} and nitrotyrosine, a reaction product of ONOO\textsuperscript{-}, were also measured, and the influence of NO synthase inhibitors, O\textsuperscript{2-} and hydroxyl radical scavengers and other effectors was examined.

Results: In BAEC cocultured with PMA-activated PMN, \textsuperscript{51}Cr release was significantly increased [14.6±2.2% at 2 h (p<0.05) and 42.6±2.7% at 4 h (p<0.01); control (nonactivated PMN), <4%]. Superoxide dismutase (100 U/ml) reduced \textsuperscript{51}Cr release to 4.6±2.2% at 2 h (p<0.05). N-Iminoethyl-L-ornithine (L-NIO, 0.1 mM) potentiated \textsuperscript{51}Cr release (30.6±3.8% at 2 h, p<0.01), and the potentiation was eliminated by anti-CD18 monoclonal antibody. The \textsuperscript{51}Cr release was completely prevented by dimethyl sulfoxide or by deferoxamine. Treatment of PMN with L-NIO inhibited NO generation and increased O\textsuperscript{2-} production. The nitrotyrosine level did not increase in BAEC cocultured with PMA-activated PMN.

Conclusion: NO-derived ONOO\textsuperscript{-} is not a major cytotoxic mediator in BAEC injury by activated PMN. NO may have a cytoprotective effect by inhibiting PMN adherence to endothelial cells.

Keywords: Nitric oxide; Superoxide; Peroxynitrite; Tyrosine nitrination; Neutrophils; Endothelial cells

1. Introduction

Endothelial cell injury mediated by polymorphonuclear leukocytes (PMN) is important in a variety of pathophysiological conditions such as inflammation and reperfusion injury. However, the mechanisms involved remain unclear. Since PMN can produce nitric oxide (NO) as well as superoxide (O\textsuperscript{2-}) [1–3] and endothelial cells [4] continuously release NO, the reaction product of NO and O\textsuperscript{2-}, peroxynitrite (ONOO\textsuperscript{-}), is generally believed to play a significant role. ONOO\textsuperscript{-} is a very potent oxidant [5,6]; it is able to oxidize protein and nonprotein sulphydryl groups [7], to mediate nitration of tyrosine residues [8,9], and to induce lipid peroxidation [10]. A recent study indicated that the ONOO\textsuperscript{-} initiated pathway is potentially more cytotoxic than the hydroxyl radical pathway in radical-induced endothelial cell injury [11]. Furthermore, NO derived ONOO\textsuperscript{-} plays a crucial role in human umbilical vein endothelial cell injury induced by PMN upon lipoxin A4 activation [12]. However, a more recent study found that ONOO\textsuperscript{-} exhibits a protective effect against ischemia-reperfusion injury through inhibition of leukocyte-endothelial cell interactions [13]; it is also well known that NO itself can inhibit the adhesion of PMN to endothelial cells [14].

In order to clarify the role of ONOO\textsuperscript{-} in conditions where three mutually reactive molecules, O\textsuperscript{2-}, NO and ONOO\textsuperscript{-}, coexist, it is necessary to use specific inhibitors or scavengers for each molecule. However, scavengers of ONOO\textsuperscript{-} such as uric acid and deferoxamine react with hydroxyl radical as well [15,16]. Furthermore, superoxide dismutase (SOD) or NO synthase (NOS) inhibitors may decrease the formation of ONOO\textsuperscript{-} concomitantly with the

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decrease of O$_2^-$ or NO, respectively. To overcome this methodological problem we used nitrotyrosine formation in BAEC cocultured with activated PMN as a measure of ONOO$^-$ induced injury, since nitrotyrosine is a stable product of ONOO$^-$ attack on tyrosine residues of cellular constituents [17]. We therefore measured nitrotyrosine content, as well as the concentrations of ONOO$^-$, NO and O$_2^-$, in the presence of various O$_2^-$ and hydroxyl radical scavengers, NOS inhibitors and other effectors in an attempt to evaluate the extent to which ONOO$^-$ mediates the endothelial cell injury by activated PMN.

2. Methods

2.1. Preparation of endothelial cells

Bovine aortic endothelial cells (BAEC) were isolated and cultured as described elsewhere [18]. Briefly, thoracic aorta, freshly obtained from a slaughterhouse, was washed by irrigating it with sterile lactate-buffered saline and incubated with 0.04% collagenase in Dulbecco’s phosphate-buffered saline (pH 7.4) containing 0.2% glucose and 16.7 mM NaHCO$_3$ at 37°C for 25 min. The digest was centrifuged at 120 g for 10 min. The pellets were washed once with cold Medium 199 and suspended in Medium 199 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were finally seeded in 25 cm$^2$ culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. The culture medium was renewed the day after isolation and every 3 days thereafter. For cell passage, the cell monolayers were detached and dispersed with trypsin (0.05%)-EDTA (0.53 mM) solution. Subcultures were grown in Medium 199 without antibiotics. Cultures from passages 6 to 12 were used for the study.

2.2. Preparation of PMN

PMN were isolated from heparinized fresh whole blood of healthy volunteers by gradient centrifugation (400 g for 30 min) using Mono-Poly resolving medium [19]. The final cell suspension contained more than 95% PMN and cell viability was more than 98% as determined by trypan blue exclusion assay.

2.3. Experimental protocol and evaluation of BAEC injury

BAEC were cocultured with PMN at the effector-to-target ratio of 10:1 in all experiments, and injury to BAEC was evaluated by $^{51}$Cr release assay as described elsewhere [20] with slight modifications. BAEC were incubated with 5 µCi of Na$_5^{51}$CrO$_4$ in 0.5 ml of culture medium in 48-well culture dishes for 20 h at 37°C and then the cells were washed three times in HBSS to remove unincorporated radioactivity. PMN were added at 7·10$^5$ cells per well and activated with 1 µM phorbol 12-myristate 13-acetate (PMA). The $^{51}$Cr release in the supernatant was assayed by aspirating 50 µl of supernatant from each well at 1, 2, 3 and 4 h and measuring the radioactivity with a gamma scintillation counter. At the end of each experiment, $^{51}$Cr remaining in BAEC was measured by lysing the cells with 0.8% Triton X-100. All values of $^{51}$Cr release were expressed as a percentage of the total incorporated $^{51}$Cr. A parallel experiment was performed in BAEC without PMN to obtain basal release and this value was subtracted from the measured release. When inhibitors were used, they were added to the PMN suspension or to the incubation medium of BAEC prior to the addition of PMN to each well.

2.4. Measurements

2.4.1. Measurement of nitrotyrosine

ONOO$^-$ nitrates cellular constituents, particularly tyrosine residues of protein or tyrosine of nonprotein origin [21]. We measured nitrotyrosine formation as an indicator of ONOO$^-$ mediated changes in cellular constituents of BAEC, using an HPLC method [22]. Briefly, BAEC cocultured with activated PMN for 2 h in buffered saline containing 1 mM Fe$^{3+}$/EDTA were hydrolyzed as previously reported with slight modifications [23]. The cells were incubated at 110°C for 24 h in a vessel containing 6 M HCl and 0.1% phenol under vacuum and then centrifuged at 7000 g for 30 min. The supernatants were analyzed by HPLC with a C18 Nucleosil column (Jasco, Tokyo, Japan) which was eluted with 50 mM KH$_2$PO$_4$–H$_2$PO$_4$ (pH 3.0) containing 10% methanol (v/v) at a flow-rate of 1 ml/min. The peaks were measured with a UV detector set at 274 nm (Jasco). The peak was identified on the basis of the retention time of authentic 3-nitro-L-tyrosine or tyrosine. Data are presented as the percentage ratio of nitrotyrosine to tyrosine.

2.4.2. Measurement of NO metabolites

Nitrite (NO$_2^-$) and nitrate (NO$_3^-$) in the supernatant from activated PMN or BAEC were measured using the Griess reagent. Briefly, the supernatant was deproteinized by heating at 100°C for 5 min, centrifuged at 3000 g for 10 min and injected into an automated flow-through spectrophotometric system as described by Green et al. [24] after reducing NO$_3^-$ to NO$_2^-$ in an on-line cadmium column (Jasco).

2.4.3. Measurement of O$_2^-$ generation from activated PMN

O$_2^-$ generation from PMN was measured with an O$_2^-$ specific chemiluminescence probe, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-alpha]pyrazin-3-one (MCLA), using a Hamamatsu C1230 photon counter with a
H-R550 photomultiplier (Hamamatsu Photonics, Hamamatsu, Japan) [26]. The measurements were performed with PMN (5×10^6 cells) in a petri dish (22 mm in diameter) in a light-shielded box. The photon counts were monitored continuously after addition of 1 μM PMA. SOD (100 U/ml) was added after the peak count was reached and O_2^- generation was expressed as SOD-inhibitable peak counts. To convert photon counts to O_2^- generation rate, the cytochrome C method was utilized.

2.4.4. Measurement of peroxynitrite

ONO^- content was measured by the use of a reported method [3], based on the principle that ONO^- nitrates 4-hydroxyphenylacetic acid (HPA) and produces NO_2^-HPA in the presence of a transition metal complex such as inactivated SOD. Cells in 24-well dishes (10^5 cells in 500 μl of HBSS–HEPES) were stimulated with 1 μM PMA in the presence of 1 mM HPA with inactivated SOD (0.1 mg/ml), after having been incubated with 100 μM 4-aminobenzoic acid hydrazide (ABAH), a myeloperoxidase inhibitor, at 37°C for 2–4 h unless otherwise indicated. The cellular supernatant was acidified with 10% H_3PO_4 and then 20% acetonitrile was added. NO_2^-HPA was measured by HPLC using a C18 reversed-phase column with acetonitrile–10% H_3PO_4 (pH 3.2) (20:80, v/v) as the mobile phase.

2.5. Reagents

Na^{51}CrO_4 was from New England Nuclear (Tokyo, Japan). PMA, deferoxamine, catalase, l-tyrosine, 3-nitro-l-tyrosine, dimethyl sulfoxide (DMSO), catalase and N^{3-nitroso-l-arginine acetate salt (L-NMMMA) were from Sigma (St. Louis, MO, USA). N^-Iminoethyl-l-ornithine (L-NIO) and N^-1-naphthylethylenediamine dihydrochloride were from Wako (Osaka, Japan). Anti-CD18 monoclonal antibody (anti-CD18 mAb) was obtained from Nichirei (Tokyo, Japan). Mono–Poly resolving medium was purchased from Dainippon (Osaka, Japan). MCLA was purchased from Tokyo Kasei (Tokyo, Japan). SOD was purchased from Tokyo (Tokyo, Japan). Mono–Poly resolving medium was purchased from Dainippon (Osaka, Japan). SOD was purchased from Tokyo Kasei (Osaka, Japan). S-Ontrosol-n-glutathione (GSNO) was purchased from Alexius (Laufen- ngen, Switzerland). S-Nitroso-N-acetyl-dl-penicillamine (SNAP) and ONOO^- were synthesized as described previously [25–27].

2.6. Statistical analysis

Results were expressed as mean±S.E. Statistical evaluation of the difference between two means was performed by using the unpaired Student’s t-test. When more than two means were compared, analysis of variance (ANOVA) followed by Tukey’s test was used.

3. Results

3.1. Effect of NOS inhibitors, SOD or anti-CD18 mAb on ^{51}Cr release from BAEC

As shown in Fig. 1. ^{51}Cr release from BAEC cocultured with nonactivated PMN remained at a low level (<4%) throughout the 4-h observation period. In BAEC cocultured with PMA-activated PMN, ^{51}Cr release was significantly increased (14.6±2.2%, at 2 h, p<0.05 and 42.6±2.7% at 4 h, p<0.01). In the presence of SOD (100 U/ml), the release was reduced significantly at all time points (4.6±1.2%, p<0.05; 11.4±1.8%, p<0.01; 23.6±2.1%, p<0.01 at 2, 3 and 4 h, respectively). In contrast, L-NIO (0.1 mM), an NOS inhibitor [28], potentiated the ^{51}Cr release at all time points (30.6±3.8%, p<0.01; 45.3±3.1%, p<0.01; 53.8±2.8%, p<0.05 at 2, 3 and 4 h, respectively). This L-NIO-potentiated ^{51}Cr release was completely blocked by pretreatment of BAEC with a monoclonal antibody directed against the adhesion molecule CD18 (4.1±1.4%, p<0.05; 3.3±1.1%, p<0.01; 8.6±2.8%, p<0.01 vs. L-NIO at 2, 3 and 4 h, respectively), which reduced the release to about the level seen with nonactivated PMN. The anti-CD18 mAb pretreatment also reduced the ^{51}Cr release to about the same level in the absence of L-NIO. Similar results were obtained in experiments with another NOS inhibitor, L-NMMA (data not shown). ^{51}Cr release increased to 37.7±2.8% at 2 h (p<
3.2. Effects of NO donors on $^{51}$Cr release

To clarify further the role of NO in PMN-mediated endothelial injury, the effect of substrates for NO generation (NO donors) was examined (Fig. 2), using SNAP (1 mM) or GSNO (1 mM). $^{51}$Cr release was significantly reduced by the addition of an NO donor [SNAP to 26.8±2.6% ($p<0.01$) and GSNO to 20.0±2.5%, ($p<0.01$) at 4 h]. This is consistent with the known anti-adhesion effect of NO and the adhesion dependence of PMN-mediated endothelial cell injury (see Section 4).

3.3. Nitrotyrosine formation in BAEC with PMN

Nitrotyrosine formation in BAEC was measured at the 2-h time point (Fig. 3). Nitrotyrosine level remained very low in the BAEC alone (28.0±12.3 μM, 0.10±0.10% of total tyrosine), in the BAEC cocultured with nonactivated PMN (32±12.1 μM, 0.125±0.05% of total tyrosine) and even in the BAEC cocultured with PMA-activated PMN (33±13.1 μM, 0.125±0.08% of total tyrosine). When BAEC were exposed to synthesized ONOO$^-$ (1 mM) as a positive control, it increased to 420 μM, 1.70±0.33% of total tyrosine. Under these conditions, the $^{51}$Cr release was comparable to that observed in BAEC cocultured with PMA-activated PMN at 4 h (61.3±8.8%). This implies that ONOO$^-$ is not a major mediator of BAEC injury by activated PMN.

3.4. Effects of deferoxamine or DMSO or catalase on $^{51}$Cr release

We next examined the contribution of hydroxyl radicals to BAEC injury (Fig. 4). Treatment with deferoxamine (0.2 mM) completely prevented the $^{51}$Cr release and DMSO at the concentration of 0.1% had a similar effect on $^{51}$Cr release. Treatment with catalase (65 U/ml) did not decrease $^{51}$Cr release at 2 h or 3 h, but decreased it slightly at 4 h (85% of that without catalase) (not shown).

3.5. NO, $O_2^-$, and ONOO$^-$ production by activated PMN and BAEC

PMA-activated PMN produced NO$_2^-$/NO$_3^-$ at a rate of...
Fig. 5. Nitrite/nitrate production from human PMN or BAEC. PMN were activated by PMA. In the l-NIO group, PMN were pretreated with l-NIO (0.1 mM) for 30 min prior to activation. Values are presented as mean±S.E. of four separate experiments. **p<0.01 compared with the PMN group, +p<0.01 compared with the PMN+PMA group.

81±17 pmol/10^6 cells/h (Fig. 5). In the presence of l-NIO (0.1 mM), the rate decreased to 39±19 pmol/10^6 cells/h. BAEC produced NO_2^-/NO_3^- at a rate of 222±43 pmol/10^6 cells/h, and this rate decreased to 44% in the presence of l-NIO. Similarly, in the presence of l-NMMA (0.1 mM) the ^51^Cr release decreased to 46% (not shown). The baseline generation of NO_2^-/NO_3^- from BAEC was not influenced by PMA (data not shown).

As shown in Fig. 6, O_2^- generation from PMA-activated PMN was 5.7±0.2 nmol/10^6 cells/min and this was increased by pretreatment of PMN with 0.1 mM l-NIO to 8.2±0.8 nmol/10^6 cells/min (p<0.05). The increase in O_2^- generation in the presence of l-NIO is in accordance with the findings of Carreras et al. and Riesco et al. [2,29]. It indicates that the O_2^- producing enzyme, NADPH oxidase, is inhibited to approximately 70% by cogenerated NO upon PMA stimulation (the inhibition of NADPH oxidase by NO in human neutrophils has been documented [30]). The direct quenching effect of NO on O_2^- is likely to be negligible, since the concentration of NO is far less than that of O_2^-.

ONO^- formation from PMA-activated PMN was 16.2±2.9 pmol/10^6 cells/h and remained unchanged in the presence of BAEC (14.7±3.5 pmol/10^6 cells/h, Fig. 7), implying that the additional NO formed by BAEC did not react with O_2^- generated by PMN. This result is puzzling, but it is possible that O_2^- released from PMN spontaneously dismutates to H_2O_2 before it reaches the NO released from BAEC.

4. Discussion

Although several reports have indicated that NOS inhibitors reduce tissue injury in various models, it is considered that the mediator of cytotoxicity is ONOO^-, not NO itself [12,31,32]. ONOO^-, formed from NO and O_2^-, is highly reactive towards protein thiol groups, tyrosine residues and phospholipids [7–10]. We have shown that ONOO^- is involved in PMN-induced cardiac myocyte injury [33], by measuring the formation of nitrotyrosine as an indicator of ONOO^- attack on tyrosine residues of cellular constituents. We also showed that the cardiac myocyte injury is markedly attenuated by the NOS inhibitor, l-NMMA. Therefore, we initially hypothesized that ONOO^- might be the major cytotoxic species in the present BAEC and PMN coculture system, since BAEC, themselves generate NO, which may result in greater production of ONOO^- than in the myocytes and PMN coculture system. However, we found that an NOS inhibitor potentiated BAEC injury (Fig. 1), that ONOO^- did not contribute substantially to BAEC injury, since nitrotyrosine formation was not observed (Fig. 4), and that O_2^- and hydroxyl radical scavengers such as SOD and DMSO attenuated the injury (Fig. 3).
As mentioned above, coculture of cardiac myocytes with PMA-activated PMN resulted in significant nitrotyrosine formation [33]. To explain the lack of nitrotyrosine formation (Fig. 4) in BAEC, despite the fact that PMN produce ONOO\(^-\) (Fig. 7), two mechanisms can be considered. First, the intracellular concentration of tyrosine residues may be lower in BAEC than in cardiac myocytes. Secondly, ONOO\(^-\) may be scavenged more efficiently in BAEC than in cardiac myocytes. The first possibility may be ruled out since the addition of synthesized ONOO\(^-\) to BAEC resulted in the formation of a significant amount of nitrotyrosine (Fig. 4). The amount of nitrotyrosine in BAEC was even higher than that of cardiac myocytes exposed to the same concentration of ONOO\(^-\) (data not shown). The second explanation is more likely since Szabo and Salzman showed that uric acid is a scavenger of ONOO\(^-\) [34] and uric acid-synthesizing enzymes such as xanthine dehydrogenase and oxidase are present at high concentrations in endothelial cells. Furthermore, it has been shown that xanthine oxidase is activated when PMN adhere to endothelial cells [35,36]. The elimination of ONOO\(^-\) by NO generated by BAEC may be another factor, since NO can react with ONOO\(^-\) [37,38]. This possibility is supported by the results in Fig. 7, which show that the formation of ONOO\(^-\) from PMN was essentially the same in the presence or absence of BAEC. That is, the additional NO generated by BAEC did not react with O\(_2\)^\(^-\) from PMN to produce additional ONOO\(^-\). This is also consistent with the finding that although nitrotyrosine staining was positive in human coronary arteries, endothelial cells appeared to be spared [21].

With regard to the aggravation of BAEC injury by NOS inhibitors, (corresponding to a protective effect of NO), this may be explained in terms of the anti-adhesion effect of NO. PMN-mediated endothelial cell injury is highly adhesion-dependent and NO is a well-known endogenous inhibitor of PMN adherence [14,39]. This is supported by the results obtained with anti-CD18 mAb, an anti-adhesion molecule mAb: \(^{31}\)Cr release was completely inhibited by anti-CD18 mAb, and in its presence, addition of NOS inhibitors did not aggravate the injury at all (Fig. 1). The mechanism underlying that NO donors (Fig. 2), which reduced the BAEC injury may be the inhibition of myeloperoxidase activity by NO donors, since NO donor markedly inhibited myeloperoxidase activity (data not shown).

We should also consider the effects of NOS inhibitors on active oxygen species. NO-induced inactivation of NADPH oxidase was demonstrated by Clancy et al. [30] and by us [40]. In the present work, we observed that l-NIO markedly increased the generation of O\(_2\)^\(^-\) from PMN (Fig. 6). This may imply that NADPH oxidase of PMN has already been inhibited by endogenously produced NO, and elimination of that inhibition by an NOS inhibitor would increase the concentration of O\(_2\)^\(^-\). O\(_2\)^\(^-\) is rapidly dismutated to H\(_2\)O\(_2\) and produces hydroxyl radical, and HOCI cytotoxic mediators in O\(_2\)^\(^-\) [20,35,41]. Thus, the inhibition of NOS may have increased the formation of hydroxyl radical and HOCI. This idea is supported by the observation that DMSO, a scavenger of both oxidants, afforded almost complete protection against endothelial cell injury by PMN (Fig. 3).

Overall, the data presented herein suggest that, although ONOO\(^-\) is generated by activated neutrophils, it is not a major mediator of cellular injury in this in vitro model.

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