Release of a leukocyte activation inhibitor by staurosporine-treated pulmonary artery endothelial cells

XILIN CHEN AND JOHN D. CATRAVAS
Vascular Biology Center, Medical College of Georgia, Augusta, Georgia 30912

Chen, Xilin, and John D. Catravas. Release of a leukocyte activation inhibitor by staurosporine-treated pulmonary artery endothelial cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L184–L192, 1998.—Bovine pulmonary arterial endothelial cells (BPAE) treated with the protein kinase C (PKC) inhibitor staurosporine inhibited O$_2^-$ generation by neutrophils exposed to phorbol myristate acetate (PMA) but did not affect O$_2^-$ generated enzymatically by xanthine/xanthine oxidase (X/XO). Similar results were obtained with conditioned medium from staurosporine-treated BPAE. The inhibitory effects of staurosporine-treated BPAE on O$_2^-$ generation were not altered by the superoxide dismutase inhibitor diethyldithiocarbamate. This BPAE-derived inhibitor was continuously released from staurosporine-pretreated BPAE for at least 5 h. The exact nature of the inhibitor remains unknown, but it appears to be a positively charged molecule with molecular weight <10,000. Treatment of either BPAE or neutrophils with staurosporine or conditioned medium from staurosporine-treated BPAE prevented the neutrophil-mediated decrease in endothelium-bound angiotensin-converting enzyme activity and cytotoxicity in BPAE. In contrast, staurosporine potentiated the H$_2$O$_2$- and X/XO-mediated endothelial cytotoxicity. These data suggest that staurosporine-treated endothelial cells release a soluble factor that inhibits neutrophil activation and protects endothelial cells from neutrophil-mediated injury.

METHODS
Isolation and Culture of Endothelial Cells

Bovine pulmonary arterial endothelial cells (BPAE) were harvested by the modified method of Ryan (23) and grown in medium 199 (Mediatech, Washington, DC) with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), penicillin (100,000 U/l), and streptomycin (100 mg/l) in T-75 flasks (Corning Glass, Corning, NY). The cultures were incubated at 37°C in a humidified atmosphere with 5% CO$_2$. The cells were identified as endothelial cells by their typical cobblestone morphology under phase-contrast microscopy, by indirect immunofluorescent staining for von Willebrand factor, and by the expression of ACE activity. The cells were grown to confluence in T-75 flasks and then subcultured into 24-well plates, the latter used for enzyme assays and neutrophil adherence experiments. All experiments with endothelial cells were performed at 2–3 days after confluence and in three- to seven-passage cells. Human brain microvascular endothelial cells were generously provided by Dr. David Hess, Department of Neurology, Medical College of Georgia (Augusta, GA).

Preparation of Neutrophil Suspensions

Neutrophils were isolated from the peritoneal cavities of adult New Zealand White rabbits with the method of O’Flaherty et al. (21). Briefly, neutrophils were obtained from peritoneal exudates 16 h after the intraperitoneal injection of 0.2% glycogen dissolved in a 0.9% NaCl solution. They were washed (centrifuged at 100 g) one time with Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ balanced salt solution (HBSS) and resuspended at a concentration of 40 × 10$^6$/ml. Three milliliters of leucocyte suspension were placed on 3 ml of Ficoll-Histo-Topaque (density 1.077; Sigma) and centrifuged at 270 g for 20 min to separate neutrophils and contaminating monocytes. The pellets (containing neutrophils) were collected and washed two times in Ca$^{2+}$- and Mg$^{2+}$-free HBSS and resuspended in Earle’s salt solution (ESS) to the appropriate neutrophil concentration. The cells obtained consisted of >95% neutrophils and were 95% viable as measured by the trypan blue exclusion test. Neutrophils were not activated by the isolation procedure, as there was no detectable increase in basal release of O$_2^-$. Monitoring of O$_2^-$ Generation

The concentration of O$_2^-$ generated by neutrophils was monitored by measuring the SOD-inhibitable reduction of ferricytochrome c. Neutrophils (0.05–1 × 10$^6$/ml) suspended in ESS were incubated with 40 mM ferricytochrome c in the...
correction for the extraction of $[^{3}H]$BPAP into toluene was performed, and the true $[^{3}H]$BPhe concentration in toluene was calculated from the solution of the equation

$$[^{3}H]BPhe = (\text{[toulen-extractable }[^{3}H]) - \frac{(f_s \times \text{total }[^{3}H]) - (f_p - f_s)}{(f_p - f_s)}$$

where $f_s$ and $f_p$ represent the fractional extraction of $[^{3}H]$BPAP and $[^{3}H]$BPhe, respectively, into toluene. Enzyme activity was calculated as

$$V_{\text{max}}/K_m = \ln \left( \frac{[S_i]}{[S]} \right) / t$$

where $V_{\text{max}}$ is maximal velocity, $K_m$ is Michaelis constant, $[S_i]$ and $[S]$ are the initial and final (surviving) substrate concentrations, respectively, and $t$ is incubation time. Enzyme activity is then expressed in units where one unit is the $V_{\text{max}}/K_m$ value equivalent to 1% substrate metabolism in 1 min under first-order reaction conditions.

Determination of Neutrophil Adherence to Endothelial Cells

Adherence of neutrophils to cultured endothelial cells was determined using the modified method of Hoover et al. (15). Briefly, postconfluent endothelial cells were rinsed two times with ESS; $^{51}$Cr-labeled neutrophils (2 mCi/ml for 1 h at 37°C) were added into each endothelial cell culture well and allowed to incubate (with or without PMA) for 1–4 h at 37°C in a 5% CO$_2$ incubator. Unattached neutrophils were then removed by aspiration, and the monolayers were rinsed one time with 0.5 ml of ESS. Adherent neutrophils and the endothelial monolayer in each well were lysed with 1 N NaOH, and the lysate plus one 0.5-ml wash were transferred to a test tube.

The radioactivity was measured in a gamma spectrometer with 95% efficiency for $^{51}$Cr. The percentage of adherent neutrophils was calculated as

$$\% \text{ adherence} = \frac{100 \times \text{cpm (adherent cells)/cpm (adherent plus nonadherent cells)}}{\text{total release}}$$

Cytotoxicity Assay

Cytotoxicity was measured by a standard $^{51}$Cr-release assay (25). The endothelial cell monolayer in a 24-well plate was incubated overnight with 2 mCi of Na$^{51}$CrO$_4$ in culture medium. Cells were then washed two times to remove unincorporated radioactivity. Suspensions of neutrophils in ESS were added to each well with or without PMA in a final volume of 1 ml. After an additional incubation at 37°C for the indicated times, the medium was removed from each well and centrifuged. The supernatant was transferred to a test tube, and the $^{51}$Cr released was quantified in a gamma spectrometer. Spontaneous release was obtained from wells receiving medium only, and total release was obtained from wells exposed to 0.2% Triton X-100. Percent cytotoxicity was calculated by the following formula

$$\% \text{cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{\text{total release}} \times 100$$

Data Analysis and Statistics

Data are presented as means ± SE of the indicated number of individual wells or tubes. Statistical comparisons between groups were performed using one-way analysis of variance followed by the Newman-Keuls test. Differences among means were considered significant at $P < 0.05$.

RESULTS

The Observation

The protein kinase C inhibitor staurosporine inhibits O$_2^-$ generation by activated neutrophils. Coincubation of neutrophils ($1 \times 10^6$/ml) with PMA (10 ng/ml) for 20 min produced a dramatic increase in O$_2^-$ generation by neutrophils, from a baseline of 2 to 18 nmol, as determined by the cytochrome c reduction assay. Treatment with staurosporine produced a concentration-dependent inhibition in O$_2^-$ generation by PMA-activated neutrophils. There was a 40% inhibition of O$_2^-$ generation at 0.1 µM staurosporine. Superoxide generation by activated neutrophils was completely inhibited at a concentration of 1 µM staurosporine (Fig. 1). Because staurosporine was dissolved in dimethyl sulfoxide (DMSO), 0.1% DMSO did not inhibit superoxide generation by activated neutrophils.

Staurosporine-pretreated BPAE inhibit O$_2^-$ generation by activated neutrophils. As shown in Fig. 2A, neutrophils ($1 \times 10^6$/ml) activated by PMA (10 ng/ml) generated 20 nmol of O$_2^-$ generation by activated neutrophils and BPAE had no effect on the O$_2^-$ generation by activated neutrophils. To investigate the effects of staurosporine-pretreated BPAE on O$_2^-$ generation by activated neutrophils, BPAE were pretreated with staurosporine (1 µM for 60 min) followed by two careful washes; staurosporine-pretreated BPAE were then co-incubated with neutrophils. Coincubation of staurospo-
rine-pretreated BPAE with neutrophils completely inhibited \( \text{O}_2^\cdot \) generation by PMA-stimulated neutrophils. Conditioned medium from staurosporine-treated BPAE inhibits \( \text{O}_2^\cdot \) generated by neutrophils exposed to PMA. To assess whether a soluble inhibitor of neutrophil activation was released by staurosporine-treated BPAE, we tested the effects of ESS conditioned by BPAE with or without staurosporine treatment on \( \text{O}_2^\cdot \) generated by neutrophils and PMA with normal medium from BPAE had no effect on \( \text{O}_2^\cdot \) generation (Fig. 2B). However, incubation of neutrophils and PMA with conditioned medium from staurosporine-pretreated BPAE totally inhibited \( \text{O}_2^\cdot \) generation by activated neutrophils (Fig. 2C). These data suggest that a soluble inhibitor released from staurosporine-pretreated BPAE inhibits \( \text{O}_2^\cdot \) generated by activated neutrophils.

Figure 3 shows the concentration response of conditioned medium from staurosporine-pretreated BPAE on \( \text{O}_2^\cdot \) generation by neutrophils exposed to PMA. There was a 40% inhibition of \( \text{O}_2^\cdot \) generation at 5% conditioned medium and up to 95% inhibition at 20% of the conditioned medium. One explanation of the observed effect of staurosporine may be that BPAE take up staurosporine during the 60-min incubation time and convert it into a more potent form of protein kinase C (PKC) inhibitor, which is then released into the medium. If this were the case, the observed inhibitory effect on neutrophil activation would be expected to decrease with time. To test this hypothesis, we treated BPAE with staurosporine (1 µM) for 60 min followed by two washes and then collected conditioned medium after each 1-h period of incubation for up to 5 h. First-hour conditioned medium was obtained by incubating ESS with staurosporine-pretreated BPAE for 1 h. Second or later hour conditioned medium was obtained by...
further incubating fresh ESS with the cells for another hour. As demonstrated in Fig. 4, staurosporine-conditioned medium exhibited similar inhibitory effects on neutrophil activation after 5 h, suggesting that the soluble inhibitor is continuously released from endothelial cells after staurosporine treatment.

Sources and Targets of the Soluble Inhibitor

The conditioned medium from staurosporine-treated BPAE also inhibited $\text{O}_2^-$ generated by mononuclear leukocytes (monocytes/macrophage 70%, lymphocytes 30%; Table 1). Furthermore, treatment of human brain microvascular endothelial cells with staurosporine (1 µM for 60 min) also inhibited superoxide generated by PMA-exposed neutrophils from 26.5 ± 8.5 to 2.5 ± 2.1 nmol.

We also tested the effects of conditioned medium from staurosporine-treated BPAE on two other neutrophil activators, A-23187 (2 µM), a calcium ionophore, and formyl-Met-Leu-Phe (FMLP; 100 nM), a plasma membrane receptor-mediated agonist. As shown in Table 1, $\text{O}_2^-$ production by FMLP- or A-23187-activated neutrophils was also inhibited by conditioned medium from staurosporine-treated BPAE.

![Figure 3](image-url)

**Fig. 3.** Concentration response of conditioned medium from BPAE treated with staurosporine (1 µM for 60 min) as reflected in the inhibition of $\text{O}_2^-$ generation by PMA (10 ng/ml)-activated neutrophils (1 × 10^6/ml). Values are means ± SE; n = 4 tubes. *P < 0.05 from control (no medium) group.

**Table 1.** Effects of conditioned medium from staurosporine-treated endothelial cells on superoxide generation by neutrophils or mononuclear leukocytes

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>n</th>
<th>Superoxide, nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>FMLP (100 nM)</td>
<td>6</td>
<td>12.5 ± 0.3*</td>
</tr>
<tr>
<td>ST-M + FMLP (100 nM)</td>
<td>4</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>A-23187 (2 µM)</td>
<td>6</td>
<td>6.3 ± 0.3*</td>
</tr>
<tr>
<td>ST-M + A-23187 (2 µM)</td>
<td>6</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>Mononuclear leukocytes (70% monocyte/macrophage and 30% lymphocytes)</td>
<td>6</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>14.5 ± 0.3*</td>
</tr>
<tr>
<td>PMA (10 ng/ml)</td>
<td>6</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>ST-M + PMA (10 ng/ml)</td>
<td>6</td>
<td>1.4 ± 0.3</td>
</tr>
</tbody>
</table>

**Effects of other PKC inhibitors on $\text{O}_2^-$ generation by neutrophils.** Two other PKC inhibitors, H-7 and sphingosine, were tested to determine whether they also might have effects similar to those of staurosporine. As shown in Fig. 5, H-7 (10–200 µM; see Ref. 4) produced a dose-dependent inhibition of $\text{O}_2^-$ by neutrophils exposed to PMA. However, conditioned medium from H-7 (200 µM for 60 min)-treated BPAE had no effect on $\text{O}_2^-$ generated by PMA-exposed neutrophils. Sphingosine (1–20 µM; see Ref. 22) also produced a dose-dependent inhibition in $\text{O}_2^-$ by PMA-exposed neutrophils; however, conditioned medium from sphingosine (1–20 µM for 60 min)-pretreated BPAE had no effect on $\text{O}_2^-$ generated by PMA-exposed neutrophils (Fig. 5).

**Characterization of the Soluble Inhibitor**

**Inhibition of arachidonate and adenosine metabolism.** The inhibition of neutrophil activation by staurosporine-pretreated BPAE was not affected by the cyclooxygenase inhibitor indomethacin (0.1 mM, 30 min before adding staurosporine and allowed to remain in the medium throughout the treatment phase), nor was it affected by diethylcarbamazine (250 µM), an inhibitor of lipoxygenase pathways (19; Table 2). Adenosine released from endothelial cells or fibroblasts has been reported to inhibit $\text{O}_2^-$ generation by FMLP-activated neutrophils (12, 13). To investigate whether the soluble
inhibitor might be adenosine. BPAE were exposed to staurosporine in the presence of adenosine deaminase. The addition of 1–5 U/ml of adenosine deaminase to BPAE treated with staurosporine for 30 min had no effect on the inhibition of neutrophil activation by staurosporine-treated BPAE (Table 2). In a separate experiment, it was also shown that adenosine (1 mM) had no effect on O₂⁻ generated by PMA-activated neutrophils.

Effects of protein synthesis inhibitors. To examine whether the soluble inhibitor might be a newly synthesized protein, the protein synthesis inhibitor cycloheximide (10 µM) was added to BPAE 30 min before staurosporine and remained in the medium for the entire treatment phase. As shown in Table 2, cycloheximide had no effect on the inhibition of neutrophil activation by staurosporine-treated BPAE.

Other characteristics. The soluble inhibitor was stable for at least 3 mo at −20°C and was resistant to protease treatment (0.1 mg/ml trypsin for 30 min), acid treatment (pH 1 for 1 h), alkaline treatment (pH 12 for 1 h), and heating (boiling for 1 h). The inhibitor was not retained by passage through an anion-exchange column [Dowex 1×8, 400-mesh (Cl⁻) anion-exchange resin] but was retained after passage through a cation-exchange column (Bio-Rad 70) and was lost from dialysis in a membrane with a molecular weight of 10,000 lower retention limit, suggesting that it is a positively charged molecule with molecular weight <10,000.

Effects of the Soluble Inhibitor of Leukocyte Activation on BPAE Function

Pretreatment of neutrophils or endothelial cells with staurosporine inhibits the neutrophil-mediated decrease in endothelial ACE activity. We have reported previously that PMA-activated neutrophils decrease endothelial ACE activity by generating oxygen free radicals (7–9). We thus investigated the effects of staurosporine on neutrophil-mediated endothelial ACE dysfunction. Staurosporine alone (1 µM) had no effect on endothelial ACE activity. When BPAE were coincubated with PMA (10 ng/ml)-activated neutrophils (1 × 10⁶/ml), endothelial ACE activity was almost totally abolished. Treatment with staurosporine produced a

![Figure 5.](http://ajplung.physiology.org/)

Table 2. Effects of different treatments on superoxide generation by neutrophils coincubated with BPAE monolayers

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>n</th>
<th>Superoxide, nmol</th>
<th>Inhibition (from PMA + PMN group), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>1.6 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>PMA (10 ng/ml) + PMN</td>
<td>4</td>
<td>19.1 ± 2.3*</td>
<td></td>
</tr>
<tr>
<td>EC-ST + PMA + PMN</td>
<td>4</td>
<td>1.8 ± 0.3</td>
<td>98.5 ± 1.7</td>
</tr>
<tr>
<td>+DTCA (1 mM)</td>
<td>6</td>
<td>1.6 ± 0.2</td>
<td>100.1 ± 1.2</td>
</tr>
<tr>
<td>+Indomethacin (0.1 mM)</td>
<td>6</td>
<td>1.4 ± 0.2</td>
<td>100.3 ± 1.2</td>
</tr>
<tr>
<td>+Diethylcarbamazine (250 mM)</td>
<td>6</td>
<td>1.7 ± 0.2</td>
<td>99.5 ± 1.1</td>
</tr>
<tr>
<td>+Cycloheximide (10 mM)</td>
<td>6</td>
<td>1.5 ± 0.2</td>
<td>100.3 ± 1.2</td>
</tr>
<tr>
<td>+Adenosine deaminase</td>
<td>6</td>
<td>1.4 ± 0.3</td>
<td>98.9 ± 1.2</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of experiments. EC-ST, staurosporine (1 µM) was preincubated with bovine pulmonary arterial endothelial cells (BPAE) for 60 min; DTCA, diethyldithiocarbamate; PMN, polymorphonuclear neutrophils. Inhibitors were incubated with BPAE 30 min before staurosporine and were also present during the staurosporine treatment period. * P < 0.05 compared with None.
dose-dependent protection; at 1 µM staurosporine, neutrophil-mediated ACE dysfunction was completely prevented (Fig. 6A). These data suggest that activation of PKC may play an important role in neutrophil-mediated endothelial ACE dysfunction. To determine the cellular source of PKC responsible for the neutrophil-mediated decrease in ACE activity, we treated either neutrophils or endothelial cells with staurosporine. Treatment of neutrophils with staurosporine for 1 h and then carefully washing out the staurosporine and coincubating treated neutrophils with PMA attenuated the neutrophil-mediated decrease in ACE activity in a dose-dependent manner similar to cotreatment of neutrophils and endothelial cells with staurosporine (Fig. 6B). However, preincubation of BPAE with staurosporine for 1 h followed by two careful washes also prevented the neutrophil-mediated decrease in endothelial ACE activity (Fig. 6C).

Staurosporine-treated endothelial cells inhibit neutrophil-mediated endothelial cytotoxicity. Because treatment of BPAE with staurosporine prevented the neutrophil-mediated decrease in endothelial ACE activity, we investigated whether it would also attenuate the activated neutrophil-mediated endothelial cytotoxicity. As shown in Fig. 7A, BPAE exposed to PMA (10 ng/ml)-activated neutrophils (4 x 10⁶/ml) for 4 h exhibited 30% cytotoxicity. Cotreatment of cultures with staurosporine (1 µM) nearly abolished the neutrophil-mediated endothelial cytotoxicity (Fig. 7A). However, pretreatment of BPAE only with staurosporine (1 µM for 60 min) followed by two washes also significantly attenuated the neutrophil-mediated endothelial cytotoxicity (Fig. 7A).

Staurosporine-treated endothelial cells potentiate H₂O₂- and X/XO-mediated endothelial cytotoxicity. In contrast to neutrophil-mediated endothelial injury, treatment of BPAE with staurosporine dramatically potentiated H₂O₂- or X/XO-induced endothelial cytotoxicity. As shown in Fig. 7B, exposure of BPAE to 0.1 mM H₂O₂ for 2 h produced 20% cytotoxicity. Treatment of BPAE with staurosporine (1 µM for 60 min) caused a threefold increase in H₂O₂-mediated cytotoxicity. Similarly, treatment of BPAE with staurosporine also potentiated the X/XO-induced endothelial cytotoxicity (Fig. 7C). These data suggest that inhibition of endothelial PKC potentiates H₂O₂- and X/XO-induced endothelial cytotoxicity.

Effects of staurosporine on adherence of PMA-activated neutrophils to endothelial cells. Neutrophil adherence to endothelial cells is an important mechanism mediating cytotoxicity. To examine whether adherence of neutrophils to endothelial cells was affected by the soluble inhibitor, we measured neutrophil adherence to staurosporine-treated BPAE. When untreated neutrophils were exposed to BPAE for 30 min, 25% of neutrophils adhered to endothelial cells (Fig. 8); this increased to 51.5% in the presence of PMA (10 ng/ml; Fig. 8). BPAE pretreated with staurosporine (1 µM for 60 min) did not affect the adherence of PMA-activated neutrophils (Fig. 8). Conditioned medium from staurosporine-treated BPAE significantly decreased adherence of PMA-activated neutrophils to BPAE (Fig. 8). Again, this conditioned medium was obtained by incubating ESS for 1 h with BPAE that had been treated with staurosporine (1 µM for 60 min) and then carefully washed two times with ESS. Exposure of BPAE to PMA and neutrophils in the presence of staurosporine (1 µM) prevented...
DISCUSSION

PMA, a strong PKC activator, is an agent frequently used to activate neutrophils and to induce acute endothelial cell injury (18, 25). We have reported previously that PMA-activated neutrophils produced a profound decrease in endothelial ACE activity in perfused rabbit lung preparation and cultured endothelial cells (7, 9). PMA-activated neutrophils produced ACE dysfunction by generating oxygen free radicals, H2O2, and its conversion to hydroxyl radicals (8). While investigating the role of PKC in the neutrophil activation and neutrophil-mediated ACE dysfunction using the PKC inhibitor staurosporine, we made the following unexpected observations:

1. Treatment of BPAE with staurosporine, like treatment of neutrophils or cotreatment of BPAE and neutrophils with staurosporine, prevents neutrophil-mediated endothelial ACE dysfunction and neutrophil-mediated endothelial cytotoxicity;
2. Treatment of BPAE with staurosporine dramatically potentiated H2O2- or X/XO-mediated endothelial cytotoxicity;
3. Conditioned medium from staurosporine-treated BPAE inhibited O2•- generation by PMA-activated neutrophils and monocytes and decreased neutrophil adherence to endothelial cells.

Our previous data suggest that neutrophil-mediated endothelial ACE dysfunction depends on PKC activity of neutrophils, since 1) preincubation of neutrophils with PMA produced a decrease in ACE activity, whereas preincubation of endothelial cells with PMA followed by incubation with neutrophils had no effects on ACE activity, and 2) inhibition of neutrophil PKC activity by staurosporine prevented neutrophil-mediated endothelial ACE dysfunction (7). However, we were surprised to find that inhibition of endothelial cell PKC activity by staurosporine also prevented the neutrophil-mediated endothelial ACE dysfunction and cytotoxicity. One pos-
sible explanation is that endothelial cell PKC activity is required for neutrophil-mediated endothelial injury. Johnson et al. (17) reported that, in isolated guinea pig lungs, the PKC inhibitor H-7 prevented pulmonary edema induced by H$_2$O$_2$, suggesting a role for PKC in H$_2$O$_2$-induced pulmonary edema. Because oxygen metabolites such as O$_2^\cdot$ or H$_2$O$_2$ are important mediators of neutrophil-mediated endothelial ACE dysfunction and cytotoxicity, we tested whether inhibition of endothelial PKC activity attenuates H$_2$O$_2$- or X/XO-induced cytotoxicity. Contrary to this hypothesis, inhibition of endothelial PKC activity dramatically potentiated the H$_2$O$_2$- or X/XO-induced cytotoxicity, suggesting that inhibition of endothelial PKC increases the vulnerability of endothelium to oxidant injury. The mechanism for this potentiation of oxidant-induced endothelial injury is not clear. One possible explanation is that inhibition of endothelial PKC activity prevents phosphorylation of certain proteins and decreases the activity of key enzymes such as antioxidant or mitochondrial enzymes, thus rendering endothelial cells more susceptible to oxidant injury.

The major discovery of this study is that endothelial cells treated with the PKC inhibitor staurosporine generate a soluble neutrophil activation inhibitor. There are several reports that have shown that coincubation of neutrophils with endothelial cells markedly inhibited the extracellular release of O$_2^\cdot$ in vitro after activation of neutrophils by membrane receptor-mediated activators such as FMLP, opsonized zymosan, or heat-killed staphylococci (2, 3, 14, 15) but not by non-plasma membrane receptor-mediated activators, e.g., PMA (2). Basford et al. (2) showed that this soluble inhibitor released by unstimulated endothelial cells appears to be a polypeptide and is not adenosine, an arachidonate metabolite, or SOD. Unlike the soluble inhibitor reported by Basford and co-workers, the inhibitor reported in this study is released only from endothelial cells treated with staurosporine and inhibits O$_2^\cdot$ generated by neutrophils activated either by a membrane receptor-mediated activator (FMLP) or a non-plasma membrane-mediated activator (PMA).

Hoover and co-workers (15) reported that O$_2^\cdot$ released at the neutrophil-endothelial interface was scavenged by endothelial SOD, accounting for a spurious “inhibition” of O$_2^\cdot$ production. Three lines of evidence suggest that scavenging did not account for our observation. First, using the X/XO system to generate O$_2^\cdot$ at levels comparable to those of PMA-activated neutrophils, we found no evidence of scavenging O$_2^\cdot$ by staurosporine-treated endothelial cells. Second, treatment of BPAE with the SOD inhibitor DTCA did not decrease the observed inhibitory effects. Third, conditioned medium from staurosporine-treated endothelial cells had qualitatively similar inhibitory effects on O$_2^\cdot$ production by neutrophils. Taken together, these observations strongly suggest that regulation of activation of neutrophils, rather than scavenging of O$_2^\cdot$ by SOD, appears to account for the inhibitory effects of conditioned medium from staurosporine-treated endothelial cells.

Two other PKC inhibitors, H-7 and sphingosine, failed to produce the same effects as staurosporine, suggesting that staurosporine’s effects may not be due to direct inhibition of PKC but instead due to some as yet unidentified mechanism. In addition to inhibiting PKC activity, staurosporine has been reported to inhibit protein tyrosine kinase activity (16, 24). Because H-7 and sphingosine do not inhibit protein tyrosine kinase, it is possible that staurosporine’s effects may be due to its ability to block protein tyrosine kinase activity.

The soluble inhibitor only partly prevented PMA-treated PMN from adhering to endothelial cells. Similarly, whereas treatment of endothelial cells with staurosporine prevented superoxide release or decrease in endothelial ACE activity from PMA-exposed PMN, it did not alter the PMA-induced increased PMN adherence to endothelial cells. One explanation is that, since staurosporine caused endothelial cell retraction, it is possible that a fraction of the PMN adhered to the subendothelial matrix rather than to endothelial cells. Although these mechanisms remain unclear, these findings suggest that the adherent PMN were not significantly activated, since they were unable to elicit either superoxide release or ACE dysfunction.

The identity of the soluble inhibitor remains elusive. Cyclooxygenase inhibitors, lipoxygenase inhibitors, adenosine deaminase, and protein synthesis inhibitors had negligible effects on its activity. It is still not clear whether or not this inhibitor is released under any physiological or pathophysiological conditions, nor do we know the mode of action of this inhibitor. Nevertheless, characterization and purification of this inhibitor may lead to discovery of a novel agent that can be used in the treatment and/or prevention of inflammatory processes.

In summary, treatment of endothelial cells with staurosporine, like treatment of neutrophils, prevented neutrophil activation as well as neutrophil-mediated endothelial ACE dysfunction and endothelial cytotoxicity. Treatment of endothelial cells with staurosporine potentiates the H$_2$O$_2$- and X/XO-mediated endothelial cytotoxicity. Endothelial cells or conditioned medium from staurosporine-treated BPAE inhibited O$_2^\cdot$ generation by activated neutrophils and increased neutrophil adherence to endothelial cells. Endothelial cells treated with staurosporine do not inhibit O$_2^\cdot$ generated by X/XO. The inhibitory effects of endothelial cells treated with staurosporine on O$_2^\cdot$ generation were not affected by the SOD inhibitor DTCA. Thus these data suggest that a soluble factor is released by endothelial cells treated with staurosporine, which can inhibit neutrophil activation and neutrophil-mediated endothelial injury.

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REFERENCES


