Nitric Oxide Inhibits Neutrophil Adhesion during Experimental Extracorporeal Circulation

Massimo Chello, M.D.,* Pasquale Mastroroberto, M.D.,* Antonietta R. Marchese, M.D.,† Giovanni Maltese, M.D.,‡ Ermenegildo Santangelo, M.D.,§ Bruno Amantea, M.D.¶

Background: Myocardial and pulmonary injuries often occur after cardiopulmonary bypass, mediated in part by neutrophil activation and adhesion to endothelial cells. The effects of nitric oxide (NO) administration on neutrophil adhesion to endothelial cells after simulated extracorporeal circulation were investigated.

Methods: Two identical extracorporeal circulation circuits were primed with fresh human blood and circulated for 2 h at 37°C. Nitric oxide at a 40-ppm concentration was added to one of the oxygenators in each pair. Neutrophil CD11b/CD18 expression and their adhesion to human umbilical vein endothelial cell monolayers were assayed in leukocytes isolated from samples drawn from the circuit 30, 60, 90, and 120 min after circulation began. In another series of experiments, blocking monoclonal antibodies to both neutrophil CD11b and CD18 were incubated with polymorphonuclear leukocytes after removal from the circuit before the adhesion assay.

Results: After 60 min of circulation, the neutrophils from NO-treated circuits showed significantly reduced CD11b/CD18 surface expression compared with the control group. There was also a significant reduction in neutrophil–endothelial adhesion in the NO group after 120 min of circulation. Monoclonal antibodies to both CD11b and CD18 significantly inhibited the adhesion of polymorphonuclear leukocytes to endothelial cells after 120 min of circulation.

Conclusions: These results confirm that neutrophil activation occurs during cardiopulmonary bypass. The addition of NO to the circuits of extracorporeal circulation significantly affects neutrophil adhesion to endothelial cells. (Key words: Adhesion; cardiopulmonary bypass; neutrophils; nitric oxide.)

CARDIOPULMONARY bypass (CPB) promotes significant activation of the complement cascade, resulting in the production of both C3a and C5a anaphylatoxins,1,2 which in turn act as potent stimulators of polymorphonuclear leukocytes (PMNs).3,4 Activation products from this reaction may be responsible for the postoperative organ dysfunction frequently observed after CPB procedures.5,6 The adhesion of neutrophils to the endothelium is directly mediated by specific "adhesion" molecules on the neutrophil and endothelial cell (EC) surfaces.7 Several studies indicate that the CD11b/CD18 complex of leukocyte adhesion receptors are involved in the adhesion of chemotactically stimulated normal human neutrophils to unstimulated human umbilical vein endothelial cell (HUVEC) monolayers in vitro.8,9 Because the extent of neutrophil activation during CPB can be responsible for postoperative morbidity,4,10 efforts to reduce this activation are of potential clinical importance. Nitric oxide (NO), produced by ECs, not only regulates vascular tone but was recently shown to play a significant role in leukocyte–endothelial interaction.10 Reduced endothelial NO production results in increased neutrophil adherence and enhanced microvascular permeability in the postcapillary venule11; furthermore, inhibition of endogenous NO formation results in a pronounced increase of PMN adherence to endothelium via upregulation of the CD11b/CD18 adhesion glycoprotein.10 Our purpose of this study was to investigate the ability of exogenous NO to modulate the CD11b/CD18-dependent adherence of neutrophil during simulated extracorporeal circulation.

Methods

Experimental Design

Blood was obtained from 15 healthy volunteers (12 men, 3 women; mean age, 38 ± 5 yr) registered at the transfusion center of our hospital. Blood was collected in heparin-processed plastic bags and used within 2 h. Informed consent for venipuncture was obtained in accord with a protocol approved by the ethics commit-
tee of the Medical School of Catanzaro. The simulated extracorporeal circuit was set up according to the description of Mellgren et al. Two identical pump circuits were each primed with 450 ml fresh whole blood mixed with 500 ml Ringer’s lactate solution. Anticoagulation was achieved with heparin to a final concentration of 4 U/ml prime. Nitric oxide in nitrogen was added at a concentration of 40 ppm to the oxygenator gas through a Y-connector immediately before the oxygenator (Dideco D-901 hollow fiber oxygenator, Dideco, Mirandola, Italy) in one of the circuits. The NO concentration in the inlet tube was recorded by the fuel cell technique (City Technology, London, UK). The other circuit served as a control. Filtered air was passed through the oxygenator at 2 l/min, and circulation commenced with a roller pump (Polystan, Værløse, Denmark) at a flow rate of 3 l/min for 2 h at 37°C. The perfusate temperature was set at 37°C. Electrolyte, glucose, and hematocrit levels were kept constant during the experiment.

To evaluate the effect of simple blood contact with plastic tubes in the absence of circulation on neutrophil activation, 80 ml blood obtained from blood donors and anticoagulated with heparin, as described before, was mixed with 90 ml Ringer’s lactate solution and used to prime a closed loop of circuit tubing (without an oxygenator) identical to those used for the experiments of simulated CPB. Blood samples were taken at baseline and then from each circuit and the closed loops 30, 60, 90, and 120 min after the circulation began. The circuits were used once and then discharged. In total, 10 experiments were performed for each group.

**Neutrophil Isolation**

Neutrophils were isolated blood drawn from the circuits using Hypaque-Ficoll density-gradient centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes, which were conducted to remove erythrocytes. Neutrophils were suspended in Hanks’ balanced salt solution, free of phenol red, Ca²⁺, and Mg²⁺, and contained 0.25% bovine serum albumin. The final cell preparation contained 98 ± 2% neutrophils. The neutrophils were then maintained on ice in Hanks’ balanced salt solution at 1–5 × 10⁶ cell/ml until they were used for the adherence assay. Isolated PMNs were >99% pure as assessed by Wright’s stained cytocentrifuge preparation and >99% viable as assessed by exclusion of trypan blue.

**Neutrophil CD11b Expression**

Neutrophil CD11b expression was detected by indirect immunofluorescence and flow cytometry, as described by Gillinov et al. Briefly, 1 ml blood was drawn from the oxygenator into a heparin-processed syringe that was kept at 0°C. Samples of 100 µl blood were placed by aliquots into polypropylene tubes and stained with anti-CD11b monoclonal antibody (mAb) CBL145 (Cymbus Biosciences, Hants, UK) as a primary reagent and with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cymbus Biosciences) as the secondary reagent. Controls included cells stained with the second reagent alone and cells stained with an irrelevant isotype-matched control mAb. No other primary mAbs were used. Erythrocytes were removed by hypotonic lysis. Flow cytometry was performed using an FACSscan (Becton Dickinson, Mountain View, CA). The granulocyte population was identified by their forward and orthogonal light-scatter characteristics. Green and red amplifier gains were calibrated with beads before each experiment (Flow Cytometry Standards Corp., Research Triangle Park, NC) to ensure that relative fluorescence values were comparable among the experiments. The mean fluorescence for each specimen was calculated from the fluorescence distribution (5,000 events) using the FACSscan Research Software, version B (Becton Dickinson). In the flow cytometry studies, the logarithmic mean fluorescence values obtained from the histograms were converted mathematically into a relative fluorescence value and expressed as a percentage increase from the observed baseline values.

**Cell Culture**

Human umbilical vein ECs were isolated from fresh umbilical cords according to the method of Jaffe. The HUVECs were plated in 75-mm plastic culture flasks and grown in medium 199 supplemented with 20% fetal bovine serum, 25 µg/ml EC growth supplement, and 90 µg/ml heparin (both from Sigma Chemical Co., St. Louis, MO) at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. All media were prepared with endotoxin-free water and filtered with Zetapore Filters (Cuno Life Sciences Division, Meriden, CT). Endotoxin-free plasticware and glassware were used in all experiments. For experimental studies, confluent HUVEC monolayers (passages 3 to 7) were trypsinized and replated on 48-well plates (Costar, Cambridge, MA) precoated with a sterile solution of 1.5% gelatin and grown to confluence for neutrophil adhesion studies.
The HUVECs were grown to confluence and used within 72 h. The identity of some endothelial cultures was checked by indirect staining with fluorescein isothiocyanate-labeled factor VIII antibody (polyclonal immunoglobulin G; Sigma Aldrich, Milano, Italy).

Polymorphonuclear Leukocyte Adherence Assay
Polymorphonuclear leukocytes isolated as described before, were fluorescence labeled with a hydrophobic fluorescence compound (3,3′-diodotadecylxocarbocyanine perchlorate (Fluka; Sigma Aldrich) as described by Lo et al.15 Cells at a concentration of 1 × 10⁶ cells/ml were incubated with 50 μg/ml Fluka in HAP buffer for 10 min at 0°C, unbound dye was removed by three washes with HAP buffer, and labeled PMNs were resuspended in medium 199 for the adhesion assay. Fluorescein-labeled PMNs (10 μl of 10⁶ cells/ml) were added to twice-washed EC monolayers. Adhesion was allowed to proceed for 15 min at 37°C, and unbound PMNs were removed by three washes with medium 199. Residual adherent PMNs on EC surfaces were counted manually, in a blinded manner, on an inverted microscope equipped for fluorescence using the filter IF355-550 at a magnification of ×100. Values of five replicates from each circuit were averaged, and the coefficients of variation between replicates were small (<10%). To examine the role of neutrophil integrins in endothelial adhesion, in another series of experiments (n = 5 for each group), neutrophils were preincubated for 15–20 min with blocking mAbs 44a (anti-CD11b, 10 μg/ml) and IBA4 (anti-CD18, 10 μg/ml), both provided by Dr. P. F. Tassone (Department of Oncology, Medical School of Catanzaro, Italy). Neutrophil-endothelial adhesion was then assayed as before.

Statistics
All values are expressed as mean ± SD. Comparisons between and within groups were made using two-way analysis of variance for repeated measures followed by the Scheffé test for multiple comparisons. The relations between independent variables were assessed by linear regression analysis. Statistical significance was set at P < 0.05.

Results

Neutrophil Count
During the perfusion period, the neutrophil count initially decreased at 30 min (control: 3,265 ± 482/mm³ to 2,161 ± 599/mm³, P < 0.05; NO: 3,394 ± 681/mm³ to 2,290 ± 678/mm³, P < 0.01) followed by progressive restitution in the ensuing 90 min of perfusion in both groups (control: 2,789 ± 605/mm³; NO: 2,961 ± 560/mm³). No significant differences in neutrophil counts were observed between the two groups at any sampling point.

Neutrophil CD11b Expression
Figure 1 shows neutrophil CD11b expression in control and treated circuits and in the closed loops. Values are expressed as a percentage of the baseline value (mean channel fluorescence: control = 82, NO = 78, closed loops = 85). In the circuits, a significant increase in CD11b expression was observed in both groups throughout the experimental period. However, 60 min after the beginning of the simulated circulation and at the different sampling points, CD11b values in the circuits added with NO were significantly less than those observed in the control circuits. In the closed loops, a significant increase in CD11b expression was observed throughout the 2 h experiment, but this was significantly lower (P < 0.05 at 30 and 60 min; P < 0.01 at 90 and 120 min) than those observed in the control extracorporeal circuits.

Polymorphonuclear Leukocyte Adhesion
Figure 2 shows the percentage of neutrophils adhering to ECs in control and treated circuits, and in the closed loops. After 60 min of simulated EC, a significant increase (P < 0.05) of neutrophil adhesion was observed in the control group, which peaked at 120 min.
complications and death, and although technical progress has improved the safety of cardiac operations, postoperative dysfunction of lung and other organs still occurs frequently.\(^4,6\) A large body of evidence suggests that the extent of neutrophil activation is correlated with the postoperative development of cardiac, renal, and pulmonary dysfunction, as well as with abnormal bleeding and pulmonary insufficiency.\(^3,4,6\) The results of the current study have two important clinical implications. First, they confirm the occurrence of PMN activation during CPB, as evidenced by both the upregulation of neutrophil CD11b/CD18 and their increased adherence to HUVEC monolayers. In this regard, our data are consistent with those of other investigators who documented increased expression of neutrophil CD11b/CD18 using a similar experimental model. Elliot and Finn\(^16\) showed an upregulation of neutrophil CD11b/CD18 after extracorporeal circulation in a simulated circuit; in a similar manner, increased neutrophil CD18 expression after CPB was also reported by Gillinov \textit{et al.}\(^15\).

The second implication of our study concerns the important role that NO plays in modulating neutrophil-endothelium interactions. Recently NO was shown to be an endogenous inhibitor of leukocyte chemotaxis adherence\(^10\) and activation.\(^17\) Inhibition of NO synthesis resulted in increased leukocyte adherence in postcapillary venules, a response that could be prevented by adding exogenous NO\(^11\) or high concentrations of L-arginine.\(^10\) The results of our study strongly support these observations, demonstrating that the administra-

\(P < 0.001\). In the circuits with NO, no significant increase in neutrophil adhesion was observed 60 min after circulation, a significant increase was recorded at 120 min \(P < 0.01\). However, neutrophil adhesion at 120 min was significantly higher in the control circuits compared with those treated with NO \(P < 0.05\). In the closed loop, a significant increase in the endothelial adhesion was observed at 90 and 120 min of circulation \(P < 0.01\) vs baseline. However, these values were significantly \(P < 0.01\) less than those observed in the control extracorporeal circuits at the corresponding sampling points.

When analyzed by linear regression analysis, no significant correlation was found between CD11b expression and neutrophil adhesion to EC monolayers.

**Polymorphonuclear Leukocyte-Endothelial Assay with Blocking Antibodies**

Figure 3 shows the results after incubation of PMNs with a CD11b antibody (clone 44a) and a CD18 antibody (clone IB4) after 2 h of circulation. Incubation of PMNs with mAb 44a caused decreases in PMN adhesion of 66% and 62% in the control and NO groups, respectively. Incubation of PMNs with mAb IB4 caused a decrease in PMN adhesion of 84% and 82% in the control and NO groups, respectively.

**Discussion**

The systemic inflammatory response that occurs in all patients after CPB remains an important cause of
tion of NO significantly reduces both CD11b/CD18 expression and PMN adherence to HUVECs.

Although our findings failed to demonstrate a direct relation between quantitative changes in the CD11b/CD18 expression and neutrophil adhesion, the results of our blocking-antibody experiments further confirm the fundamental role that neutrophil integrins, particularly CD11b, play in the adhesion of neutrophils to ECs. Furthermore, in our study we only evaluated quantitative changes in CD11b neutrophil expression. As suggested by Gillinov et al., CD11b/CD18 quantitative upregulation is not an absolute requirement for adhesive interactions between neutrophils and vascular endothelium, whereas qualitative alterations in the CD11b/CD18 molecule may be more important to neutrophil-endothelial adhesion than quantitative modifications. In addition, other neutrophil adhesion molecules, including the selectins, as well as specific endothelial adhesion molecules (ELAM-1, ICAM-1), may mediate neutrophil adherence to endothelium without participation of Mac-1.

Recent data suggest that one potential mechanism of the anti-adhesive effects of NO is through its role as a free-radical scavenger. Nitric oxide may also decrease leukocyte-endothelial interactions by decreasing the production or release of stimuli, which can activate either the endothelium or the leukocyte. In this regard, Heller et al. showed that NO donors directly inhibit the production of platelet-activating factor, a well-known activator of PMN rolling, by human ECs exposed to thrombin. Alternately, NO may directly inhibit the activation of polymorphonuclear cells. Moilanen et al. suggest that exogenous NO donors increase guanosine 3′,5′-cyclic monophosphate within the PMNs. These investigators found that NO donors inhibited chemotaxis and reduced the oxidative burst of PMNs.

**Study Limitations**

Our conclusions are limited because the experiments were performed in a simulated EC and not in a human EC. However, the advantages and disadvantages of this model for assessing neutrophil activation have already been discussed by Moat et al.

In the current study, changes in neutrophil adherence were studied in an experimental model of cultured HUVECs. At least two considerations are mandatory. First, although the adherence assay that we used is reliable with regard to that specific vascular bed, nevertheless, it should be emphasized that it is not possible to affirm whether similar changes also occur with systemic endothelium. Therefore, further examination of endothelium from other clinically important vascular beds might be worthwhile.

Second, once microvascular cells from any specific organ are placed into culture and passaged, the differences between cells of one organ and another may become blurred, as cell phenotypes change in response to the *in vitro* conditions during passage. Furthermore, concerns that isolation procedures, particularly the effect of temperature, may alter neutrophil function have been reported.

Finally, in our study, which used an artificial extracorporeal system, we did not measure plasma nitrate concentration in samples from the circuits. However, using a similar artificial system, Mellgren et al. found a significant increase in plasma nitrate in samples from circuits added with NO. Because previous studies showed that inhaled NO is metabolized to nitrate in erythrocytes *in vivo*, the accumulation of nitrate observed in their study indicates that NO added to the sweep gas in fact entered the blood through the oxygenator, and furthermore that it was metabolized in the blood perfusing the extracorporeal system in the same way as it is *in vivo*.

In conclusion, although further studies are needed to clarify the exact mechanism of action, from our data we can affirm that exogenous NO added to the extracorporeal circuits inhibits leukocyte adhesion to the venous endothelium after CPB.

**References**


8. Smith CV, Marlin SD, Rothlein R, Toman C, Anderson GC: Coop-


