Mechanisms of Amelioration of Glucose-Induced Endothelial Dysfunction Following Inhibition of Protein Kinase C In Vivo

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Inhibition of protein kinase C (PKC) activity has been shown to improve the endothelial dysfunction associated with hyperglycemia and diabetes. The mechanisms by which inhibition of PKC activity ameliorates endothelial dysfunction in diabetes are not well understood. We investigated the relationship between PKC inhibition and leukocyte-endothelium interaction in the microcirculation of the rat mesentery exposed to 25 mmol/l D-glucose for 12 h. D-Glucose significantly increased leukocyte rolling and adherence in mesenteric postcapillary venules. This proinflammatory action of D-glucose was inhibited by superfusion of the mesentery with 30 nmol/l bisindolylmaleimide-I, a potent, selective PKC inhibitor ($P < 0.01$ vs. glucose alone after 90 min of superfusion). Immunohistochemical localization of the cell adhesion molecules P-selectin and intercellular adhesion molecule (ICAM)-1 on the endothelial cell surface was increased by 25 mmol/l D-glucose ($P < 0.001$ vs. control tissue from rats injected with saline), which was significantly reduced by bisindolylmaleimide-I ($P < 0.001$ vs. glucose alone). In addition, we studied adhesion of isolated neutrophils to rat superior mesenteric artery (SMA) vascular segments stimulated with 25 mmol/l D-glucose for 4 h in vitro. Pretreatment of the SMA vascular segments with either superoxide dismutase enzyme (100 units/ml) or bisindolylmaleimide-I (30 nmol/l) equally inhibited the increased neutrophil adherence to SMA endothelium in response to glucose. These data demonstrate that inhibition of PKC activity reduces leukocyte-endothelium interactions by suppressing surface expression of endothelial cell adhesion molecules in response to increased oxidative stress. These results provide a novel mechanism by which inhibition of PKC activity improves endothelial cell function in hyperglycemia and diabetes. *Diabetes* 51: 1556–1564, 2002

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Received for publication 31 May 2001 and accepted in revised form 24 January 2002.

ICAM, intercellular adhesion molecule; K-H, Krebs-Henseleit; MABP, mean arterial blood pressure; NO, nitric oxide; NOS, NO synthase; PKC, protein kinase C; PMN, polymorphonuclear leukocyte; RBC, red blood cell; SMA, superior mesenteric artery; SOD, superoxide dismutase.

Endothelial dysfunction has been shown to play a key role in the pathophysiology of macrovascular and microvascular complications of diabetes (1,2). Although the exact mechanisms of diabetes-induced endothelial dysfunction are unknown, numerous studies have implicated increased protein kinase C (PKC) activity in the pathophysiology of diabetic vasculopathy. Thus, PKC and its activating cofactor diacylglycerol (DAG) are elevated in several organs in diabetes (3,4) and in normal tissues exposed to high glucose (5). Pathological elevations of glucose levels per se are sufficient to impair endothelial function of large arteries (6), capillaries (7), and mesenteric venules (8). This inflammatory response to glucose can be ameliorated by inhibition of PKC activity (8). Inhibition of PKC activity attenuates vascular dysfunction also in diabetes, a pathophysiologic condition more complex than hyperglycemia alone (9).

Recent studies investigating the role of PKC in glucose-induced endothelial dysfunction have suggested that inhibition of PKC activity increases availability of endothelium-derived nitric oxide (NO) (10) and attenuates leukocyte entrapment in the retinal microcirculation of diabetic rats (11). NO exerts strong anti-inflammatory effects by inhibiting the expression of cell adhesion molecules in the vascular endothelium (12). Cell adhesion molecules expressed on endothelial cells and circulating leukocytes regulate leukocyte-endothelium interaction during inflammation (13). In this regard, it has been demonstrated that exposure of the vascular endothelium to elevated ambient glucose induces expression of the cell adhesion molecules P-selectin and ICAM-1 both in vitro (14) and in vivo (15). Increased expression of endothelial cell adhesion molecules in response to glucose has also been correlated with loss of NO production (15) and activation of the nuclear transcription factor-κB by increased oxidative stress (14).

In the present study, we tested the hypothesis that inhibition of PKC activity would exert anti-inflammatory effects in the setting of acute hyperglycemia by preventing upregulation of endothelial cell adhesion molecules in the microcirculation. Using a well-established rat model of intravital microscopy, we studied the effects of PKC inhibition on leukocyte rolling and adherence, as well as on surface expression of P-selectin and intercellular adhesion molecule (ICAM)-1, in the rat mesenteric microcirculation exposed to elevated ambient glucose. To further investigate the mechanism of the protective action of PKC

inhibition on vascular endothelial function, we also studied the role that attenuation of oxidative stress and release of endothelial NO play in the anti-inflammatory effect of PKC inhibition.

RESEARCH DESIGN AND METHODS

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All animal protocols have also been approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Animal models and study protocols used for intravital microscopy

Local hyperglycemia of the rat mesentery. The rat mesenteric microvasculature was exposed to elevated ambient glucose according to an experimental model of local hyperglycemia recently described by our laboratory (15). Briefly, male Sprague-Dawley rats (ACE, Boyertown, PA) weighing 275–300 g were injected intraperitoneally with a single 3-ml dose of either 5 mmol/l D-glucose solution (normal glucose concentration) or 25 mmol/l D-glucose solution (hyperglycemia) 12 h before the beginning of the experiment. In this regard, we have previously demonstrated that a single intraperitoneal injection of 25 mmol/l D-glucose to the rat elevates the glucose content of the peritoneal fluid and upregulates leukocyte-endothelium interactions in the mesenteric microcirculation, in the absence of any significant change in circulating levels of glucose and insulin in the blood (15).

In the present study, rats were randomly divided into one of six groups: *1*) saline-injected rats superfused with Krebs-Henseleit (K-H) buffer; *2*) saline-injected rats superfused with 30 nmol/l bisindolylmaleimide-I; 3) 5 mmol/l D-glucose–injected rats superfused with K-H buffer; 4) 25 mmol/l D-glucose–injected rats superfused with K-H buffer; 5) 25 mmol/l D-glucose–injected rats superfused with 30 nmol/l bisindolylmaleimide-V; or 6) 25 mmol/l glucose–injected rats superfused with 30 nmol/l bisindolylmaleimide-I.

In additional control experiments, six rats were injected intraperitoneally with 3 ml of 25 mmol/l L-glucose solution to exclude nonspecific osmotic effect of glucose on leukocyte-endothelium interaction. L-Glucose failed to increase leukocyte rolling and leukocyte adherence in rat postcapillary venules (NS vs. control mesenteries superfused with K-H buffer alone).

Bisindolylmaleimide-I and its inactive control bisindolylmaleimide-V, which lacks PKC-inhibiting properties, were obtained from Calbiochem (La Jolla, CA). Bisindolylmaleimide-I is a cell-permeable PKC inhibitor (half-maximal inhibitory concentration [K_i], 10 nmol/l) that shows high selectivity for PKCα, β_1, β_2, γ, and ϵ isozymes. Stock concentrations of bisindolylmaleimides were prepared in DMSO and further diluted in K-H buffer for intravital microscopy experiments. The final content of DMSO in the K-H buffer was $<$0.01%; at that concentration, DMSO does not affect leukocyte-endothelium interaction as assessed in control experiments (data not shown).

Intravital microscopy of rat mesenteric venules. On the day of the experiment, rats were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Additional sodium pentobarbital was administered intraperitoneally as needed during the 2-h intravital microscopy observation period. The left carotid artery was cannulated using a polyethylene catheter for measurement of mean arterial blood pressure (MABP). A loop of ileal intestine was exteriorized through a midline incision and placed in a temperature-controlled, fluid-filled Plexiglas chamber for observation of the mesenteric microcirculation by intravital microscopy, as previously described (16). The mesentery was then superfused with a modified K-H buffer (warmed to 37°C and bubbled with 95% nitrogen and 5% carbon dioxide). MABP was monitored and recorded using a Grass model 7 oscillograph recorder with a Statham P23AC pressure transducer (Gould Nicolet Technologies, Ilford, UK). Erythrocyte velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute, College Station, TX) as previously described (17). Venular shear rates (g) were calculated using the venular diameter (D) and red blood cell (RBC) velocity (V) with the following formula: $g = 8(V_{mean}/D)$, where $V_{mean} = V/1.6$.

After 20 min of stabilization time, a 20- to 35-μm-diameter venule was chosen for observation of leukocyte-endothelium interactions. A baseline reading was taken to establish basal values for leukocyte rolling and leukocyte adherence. Readings were subsequently taken 30, 60, 90, and 120 min after the baseline readings to quantify leukocyte rolling and adherence. Video recordings were made using a video camera and videocassette recorder.

Immunohistochemistry. After completion of intravital microscopy experiments, the superior mesenteric artery (SMA) and the portal vein were cannulated for perfusion of the small bowel as previously described (16). Briefly, the ileum was first washed free of blood by perfusion with K-H buffer (warmed to 37°C and bubbled with 95% O_2 and 5% CO_2). Once the venous perfusate was free of blood, perfusion was initiated with iced 4% paraformaldehyde mixed in phosphate-buffered 0.9% NaCl for 5 min. A 3- to 4-cm segment of ileum was isolated from the perfused intestine and fixed in 4% paraformaldehyde for 90 min at 4°C. Tissue sections were embedded in plastic (Immunobed; Polysciences, Warrington, PA), and 4-μm-thick sections were cut and transferred to Vectabond coated slides (Vector Laboratories, Burlingame, CA). Immunohistochemical localization of P-selectin and ICAM-1 was accomplished by the avidin/biotin immunoperoxidase technique (Vectastain ABC Reagent; Vector Laboratories) and using monoclonal antibodies against P-selectin (PB1.3) and ICAM-1 (Genzyme, Cambridge, MA) expressed on the endothelial cell surface. PB1.3 is a monoclonal antibody that recognizes only cell surface–expressed P-selectin (18). A total of 50 venules were analyzed per tissue section, 9 sections were examined per rat, and the percentage of positive staining was determined.

Adhesion of rat neutrophils to SMA endothelium in vitro

Isolation of polymorphonuclear leukocytes. Donor Sprague-Dawley rats (350–400 g) were anesthetized with pentobarbital sodium 60 mg/kg i.p.. Polymorphonuclear leukocytes (PMNs) were isolated from rat blood by modification of the method of Williams using the hetastarch exchange transfusion method (19). Briefly, a three-way stopcock connected to a cannula placed in the femoral artery was used to alternately collect blood and infuse 6% hetastarch (Baxter, Deerfield, IL)/25 units/ml heparin (Elkins-Sinn, Cherry Hill, NJ). Each 2-ml aliquot of blood was mixed with 2 ml hetastarch until a final volume of 50 ml was collected. Erythrocytes were sedimented with gravity for ~40 min. The supernatant was underlayered with *N*,*N*′-bis(2,3-dihydroxpropyl)-5-[*N*-(2,3-dihydroxypropyl) acetamido]-2,4,6-triiodo-isophthalamide (Nycomed Pharma, Oslo, Norway) and centrifuged at 300*g* for 30 min. The supernatant was discarded, and the pellet containing RBCs and PMNs was lysed with a hypotonic solution containing NH_4Cl (1.5 mol/l), $NaHCO_3$ (100 mmol/l), and disodium EDTA (10 mmol/l), pH 7.4. After lysing the RBCs, the pellet was washed three times with 10 mmol/l PBS.

PMN adherence. SMAs were removed from donor rats, freed from fat and connective tissue, and cut into 2- to 3-mm-wide sections. SMA segments were opened and placed endothelial cell surface up in a cell culture dish containing 3 ml K-H buffer alone (control) or 3 ml of 25 mmol/l D-glucose K-H buffer. The SMA tissue was then incubated for 4 h in a metabolic shaker culture bath at 37°C to activate the endothelium by inducing surface expression of adhesion molecules. During the 4-h incubation time, the control K-H buffer and the high-glucose buffer in the tissue bath were replaced every 20 min. We have previously demonstrated that a 4-h incubation time is required for upregulation of leukocyte-endothelium interaction under hyperglycemic conditions (15). The glucose-activated endothelium was then coincubated with fluorescent dye–labeled PMNs (4×10^5 cells/ml) for 30 min as previously described (20). In some experiments, 100 units/ml superoxide dismutase (SOD) (Sigma, St. Louis, MO), the PKC inhibitor bisindolylmaleimide-I (30 nmol/l), or 100 units/ml SOD plus 30 nmol/l bisindolylmaleimide-I were added to the incubation bath 30 min before the addition of neutrophils to study the role of superoxide release and inhibition of PKC activity on adhesion of neutrophils to the SMA endothelium under hyperglycemic conditions. The number of adherent PMNs was counted by epifluorescence microscopy. Five different fields of each endothelial surface were counted from each vascular segment, and the results are expressed as adherent PMNs per square millimeter of endothelium.

Quantification of NO released from isolated aortic segments. Freshly isolated rat aortic tissue was used as the source of primary endothelial cells. Thoracic aortas were rapidly isolated from donor rats. Aortas were immersed in warm oxygenated K-H solution and cleansed of adherent fat and connective tissue. Rings of 6–7 mm in length were subsequently cut and opened from randomly selected areas of the aorta, and finally fixed by small pins, with the endothelial surface up, in 24-well culture dishes containing 1 ml K-H solution. After equilibration at 37°C, basal NO released into the K-H solution was measured as previously described (15), using an internally shielded polarographic NO electrode connected to a NO meter (Iso-NO; World Precision Instruments, Sarasota, FL). Once the basal release of NO was measured, the aortic segments were washed three times with K-H buffer and then incubated with 30 nmol/l bisindolylmaleimide-I for 30 min to measure the effect of PKC inhibition on basal release of NO. Calibration of the NO electrode was performed daily before each experimental protocol (15).

Data analysis. All data are presented as means ± SE. Data were compared by ANOVA with post-hoc analysis by Fisher's corrected t test. All data on leukocyte rolling and adherence, as well as on MABP and shear rates, were analyzed by ANOVA for repeated measurements. Probabilities of 0.05 or less were considered statistically significant.

RESULTS

Rat intravital microscopy. There were no significant differences in MABP values among any of the groups

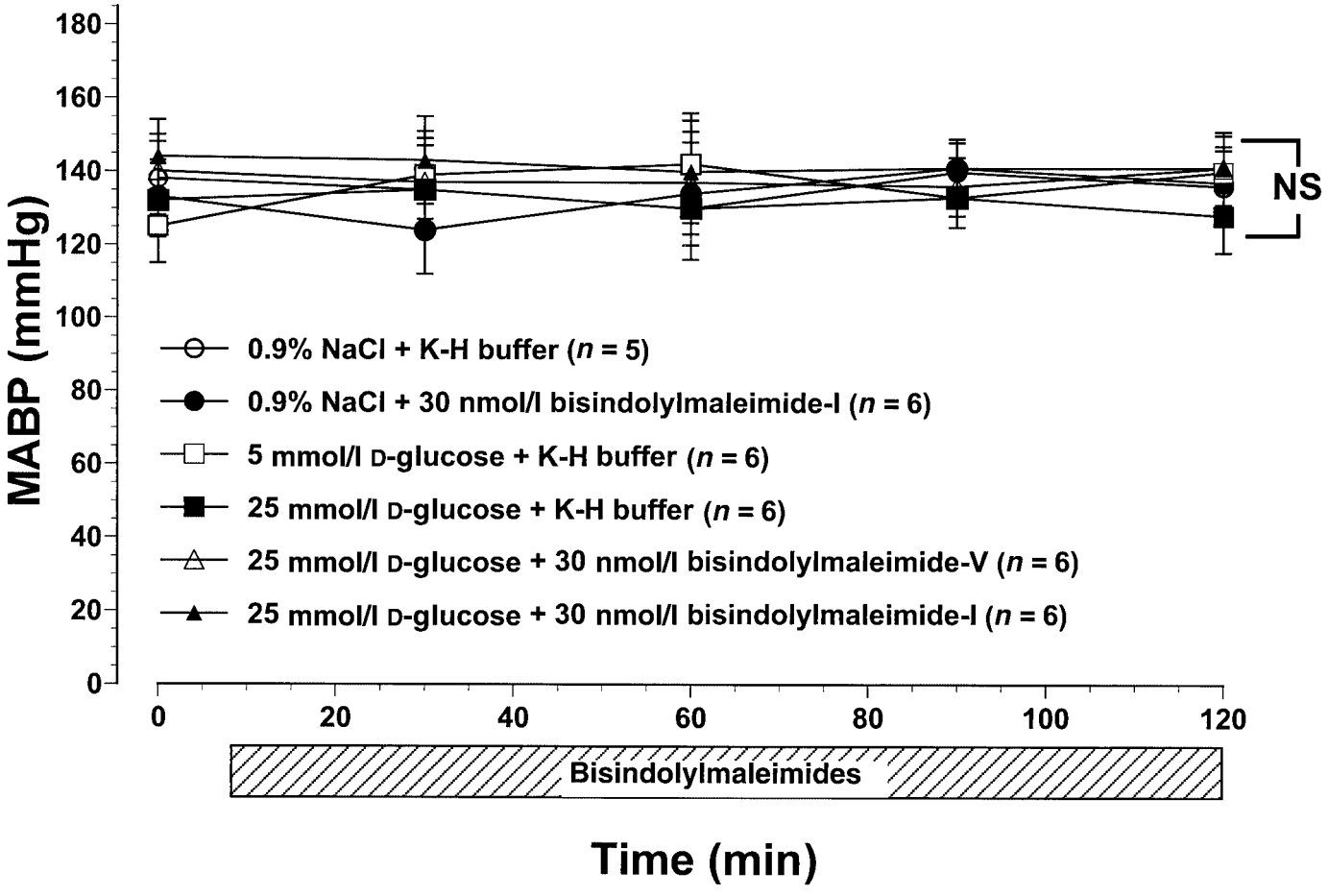

FIG. 1. Time course of MABP in rats given intraperitoneal injection of saline or 25 mmol/l D-glucose and superfused with K-H buffer alone or K-H buffer containing 30 nmol/l bisindolylmaleimide-I. All values are means ± SE. MABP values were not significantly different among any of the groups studied.

studied (Fig. 1). Following intraperitoneal glucose injection, shear force values were maintained in the physiological range (22), despite being significantly reduced (Table 1). These effects of hyperglycemia on venular shear rates are consistent with previous data showing comparable values in rat mesenteric venules following induction of experimental diabetes (23). Superfusion of the mesenteric tissue with bisindolylmaleimide-I (Table 1) did not change venular shear rate values significantly. Thus, neither a 12-h exposure to elevated ambient glucose nor bisindolylmaleimide-I superfusion affected leukocyte-endothelial dynamics in the rat mesenteric microcirculation by changing local rheological properties beyond the physiologic range.

Leukocyte rolling in postcapillary venules of rats injected with 25 mmol/l D-glucose was stable over the entire observation time, with a leukocyte flux of ~65 white blood cells/min (Fig. 2). These values represent an approximately sixfold increase in leukocyte rolling compared with values observed in control venules from saline-injected rats and rats injected with 5 mmol/l D-glucose (normal glucose concentration). Superfusion of the mesenteric tissue with 30 nmol/l bisindolylmaleimide-I significantly attenuated the inflammatory action of D-glucose (Fig. 2).

Figure 3 illustrates leukocyte adherence to the venular endothelium of the rat mesentery exposed to elevated ambient glucose. Very low numbers of adherent leuko-

TABLE 1
Venular diameter and venular shear rates in rat mesenteric venules stimulated with 25 mmol/l D-glucose for 12 h and superfused with K-H alone or K-H buffer containing 30 nmol bisindolylmaleamides

Group	n	Venular diameter (μm)	Venular shear rate		
			Baseline (0 min)	60 min	120 min
0.9% NaCl + Krebs-Heseleit	6	26.6 ± 1.9	674 ± 48*	767 ± 82*	798 ± 31*
0.9% NaCl + 30 nmol/l bisindolylmaleamide-I	6	25.7 ± 1.1	865 ± 113*	825 ± 75*	795 ± 96*
25 mmol/l D-glucose	6	32.1 ± 0.9	499 ± 32	484 ± 38	447 ± 23
25 mmol/l D-glucose + 30 nmol/l bisindolylmaleamide-V	5	32.0 ± 0.9	537 ± 51	494 ± 33	495 ± 56
25 mmol/l D-glucose + 30 nmol/l bisindolylmaleamide-I	6	30.0 ± 1.8	533 ± 57	543 ± 105	592 ± 112

Data are means ± SE. *$P < 0.01$ vs glucose-injected groups.

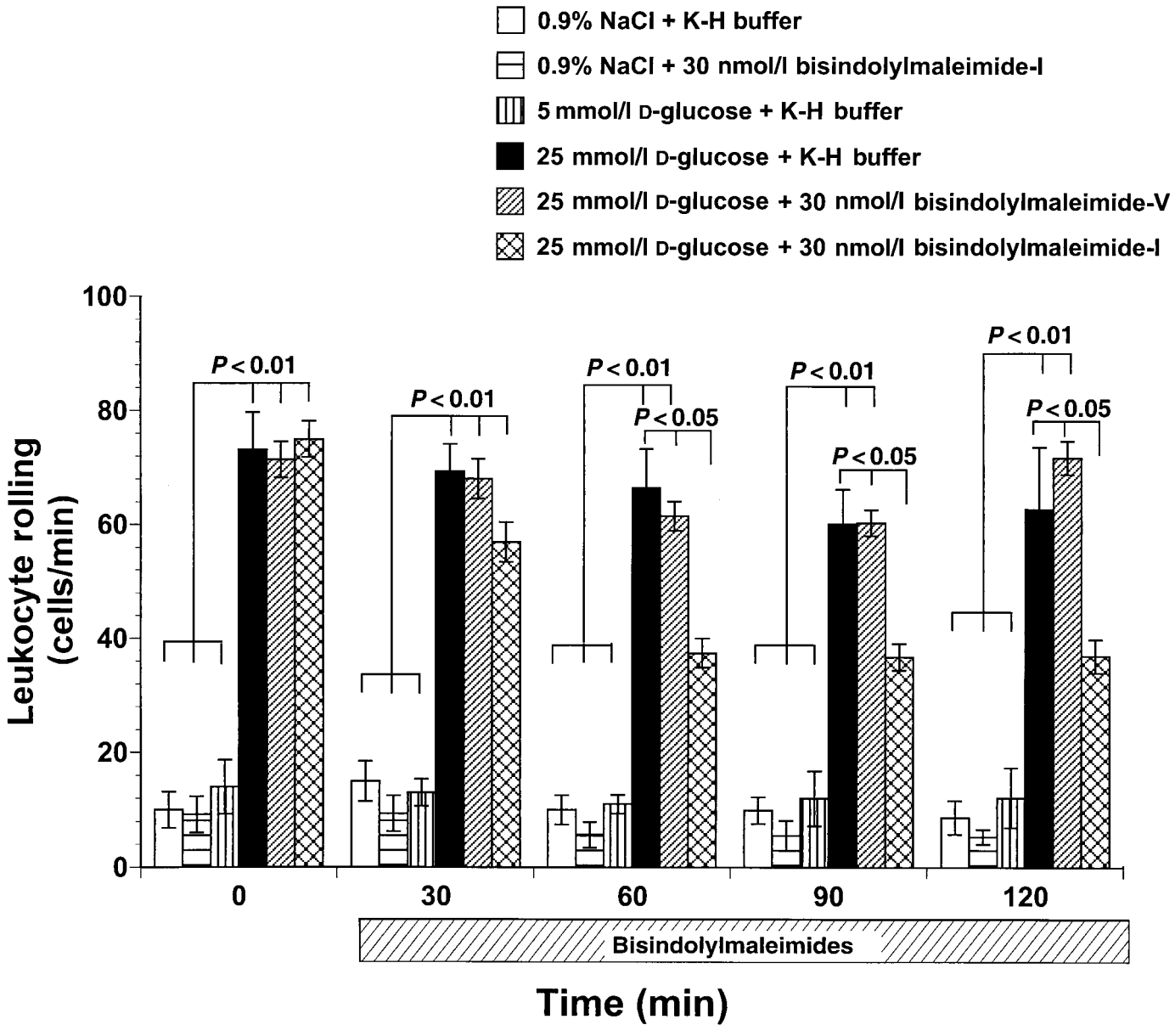

FIG. 2. Effects of bisindolylmaleimide-I and its inactive control compound bisindolylmaleimide-V on leukocyte rolling studied in rat mesenteric venules exposed to 25 mmol/l D-glucose for 12 h. Bisindolylmaleimide-I was superfused on the exposed mesentery at a concentration of 30 nmol/l. Bar heights represent mean values; brackets indicate ± SE.

cytes were recorded in mesenteric venules from control rats or rats injected with 5 mmol/l D-glucose (Fig. 3). Intraperitoneal injection of 25 mmol/l D-glucose to rats caused a fourfold increase in the number of leukocytes that adhered to the venular endothelium ($P < 0.01$ vs. rats injected with saline or 5 mmol/l D-glucose) (Fig. 3). Superfusion of the mesentery with 30 nmol/l bisindolylmaleimide-I completely blocked the effects of glucose on leukocyte adherence ($P < 0.01$ vs. glucose alone) (Fig. 3).

To exclude potential nonspecific actions of bisindolylmaleimide-I on leukocyte-endothelium interaction in the rat mesenteric microcirculation, we also studied leukocyte rolling and adherence after superfusion of the rat mesentery with bisindolylmaleimide-V, an inactive bisindolylmaleimide structural analog that lacks PKC-inhibiting properties. Interestingly, at the concentration of 30 nmol/l, bisindolylmaleimide-V was unable to attenuate glucose-induced leukocyte rolling (Fig. 2) and adherence (Fig. 3), thus confirming that the pharmacological properties observed for the active bisindolylmaleimide-I analog were not due to nonspecific interaction of its molecular structures with biological systems.

Thus, the PKC inhibitor bisindolylmaleimide-I effectively attenuated leukocyte-endothelium interactions in rat mesenteric venules exposed to elevated ambient glucose for 12 h.

Immunohistochemistry. Endothelial surface expression of the cell adhesion molecule P-selectin is illustrated in Fig. 4. The percentage of venules staining positively for P-selectin in ileal sections from control rats injected with saline was consistently low at 20% (Fig. 4). However, intraperitoneal injection of 25 mmol/l D-glucose to the rat caused a threefold increase in P-selectin expression on the venular endothelium as quantified by immunostaining

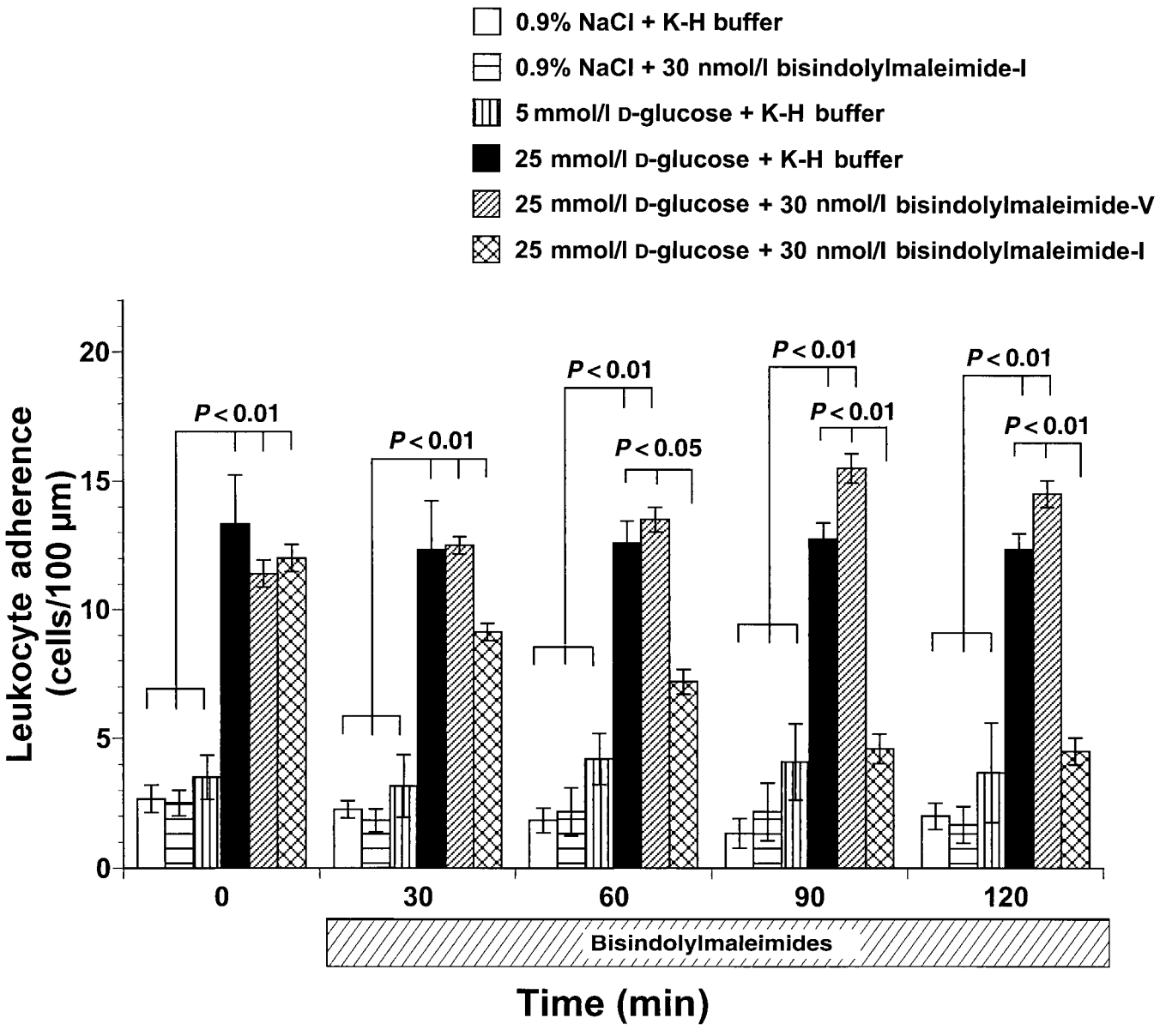

FIG. 3. Effects of bisindolylmaleimide-I and its inactive control compound bisindolylmaleimide-V on leukocyte adherence studied in rat mesenteric venules exposed to 25 mmol/l D-glucose for 12 h. Bisindolylmaleimide-I was superfused on the exposed mesentery at a concentration of 30 nmol/l. Bar heights represent mean values; brackets indicate ± SE.

($P < 0.001$). This increase in endothelial P-selectin expression was significantly attenuated by superfusion of the mesentery with 30 nmol/l bisindolylmaleimide-I for 2 h ($P < 0.001$ vs. glucose alone) (Fig. 4). These data are consistent with attenuation of glucose-induced leukocyte rolling, following superfusion of the mesentery with bisindolylmaleimide-I.

Expression of the endothelial cell adhesion molecule ICAM-1 is also illustrated in Fig. 4. The percentage of venules staining positively for ICAM-1 in ileal sections from control rats receiving saline was ~30% (Fig. 4). Exposure of the rat mesentery to 25 mmol/l D-glucose significantly upregulated ICAM-1 expression on the venular endothelium ($P < 0.01$). This increase in expression of ICAM-1 was significantly attenuated by superfusion of the mesentery with 30 nmol/l bisindolylmaleimide-I for 2 h ($P < 0.01$) (Fig. 4). Thus, inhibition of PKC activity inhibits endothelial cell surface expression of two important cell adhesion molecules, P-selectin and ICAM-1, in response to elevated ambient glucose. This appears to be a key mechanism of the anti-inflammatory action of PKC inhibition during hyperglycemia.

Adherence of PMNs to isolated rat SMA segments. To investigate the relationship among oxidative stress, PKC activity, and leukocyte-endothelium interactions, we studied the adherence of isolated rat neutrophils to rat vascular endothelium exposed to 25 mmol/l D-glucose for 4 consecutive hours. Very few rat PMNs adhered to control SMA segments in vitro (Fig. 5). In contrast, PMN adherence to the endothelium of isolated rat SMA segments was increased 2.5-fold by 25 mmol/l D-glucose (Fig. 5). Incubation of the SMA tissue with 100 units/ml SOD blocked the increase in neutrophil adherence induced by glucose ($P < 0.001$) (Fig. 5). A comparable degree of inhibition of

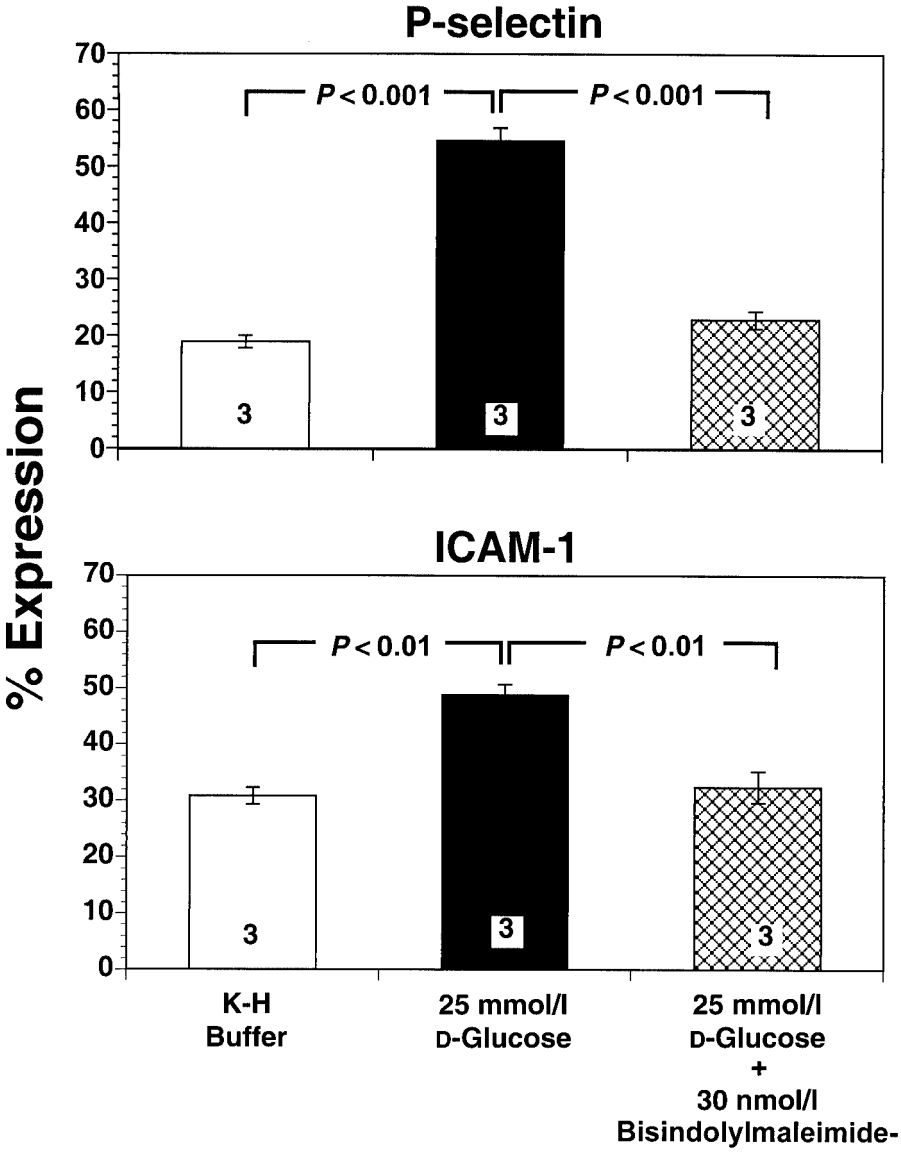

FIG. 4. Immunohistochemical localization of P-selectin and ICAM-1 on the endothelial surface of ileal venules fixed after intravital microscopy experiments. Sections were probed with primary antibodies to P-selectin and ICAM-1 and then stained using the immunoperoxidase system. Values are percentages of venules whose circumference is at least 50% stained with brown peroxidase. Bar heights represent mean values; brackets indicate ± SE. Numbers at base of bars indicate the numbers of rats studied in each group. Nine sections were studied in each rat, and 50 venules were counted in each section.

neutrophil adhesion was obtained following incubation of the SMA endothelium with 30 nmol/l bisindolylmaleimide-I ($P < 0.001$ vs. control) (Fig. 5). Concomitant incubation of the SMA segments with 100 units/ml SOD and 30 nmol/l bisindolylmaleimide-I did not synergize the inhibitory effect of bisindolylmaleimide-I on neutrophil adherence, thus suggesting that inhibition of PKC activity attenuated neutrophil adherence mainly by reduced oxidative stress. Finally, incubation of SMA rings with the inactive analog bisindolylmaleimide-V failed to inhibit glucose-induced neutrophil adhesion (16 ± 3 PMNs/mm^2; NS vs. 25 mmol/l D-glucose alone).

These data strongly indicate that PKC is involved in the mechanism by which oxidative stress upregulates leukocyte-endothelium interactions during hyperglycemia.

Bisindolylmaleimide-I increases basal release of NO from isolated rat aortic segments. We detected a small level of basal NO release in the range of 25 ± 5.3 nmol/mg of tissue in aortic rings isolated from control rats and incubated with K-H buffer (Fig. 6). Thirty minutes after the aortic segments were incubated with 30 nmol/l bisindolylmaleimide-I, the basal release of NO doubled ($P < 0.01$ vs. control). In contrast, aortic rings incubated with 30 nmol/l of the inactive PKC inhibitor bisindolylmaleimide-V did not show significant increased release of NO (Fig. 6). Therefore, inhibition of PKC activity increases release of endothelium-derived NO in the vascular endothelium.

DISCUSSION

In the present study, acute endothelial dysfunction, as evidenced by increased leukocyte rolling and adherence, was induced in the rat mesenteric microcirculation by a single intraperitoneal injection of 25 mmol/l D-glucose. This inflammatory response was blocked by nanomolar concentrations of bisindolylmaneimide-I, a specific inhib-

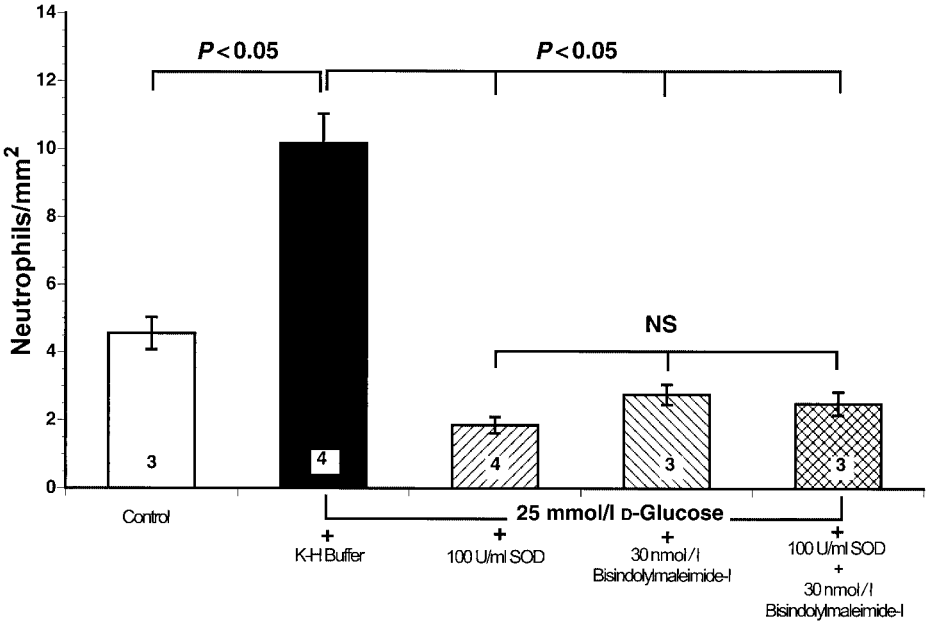

FIG. 5. Neutrophil adherence to isolated segments of rat SMA. Neutrophils were isolated from autologous blood and fluorescence-labeled with the P2KH linker system (Sigma, St. Louis, MO). Bisindolylmaleimide-I significantly attenuated the number of neutrophils adhering to SMA endothelium activated with 25 mmol/l D-glucose. Similar results were obtained with SOD. Bar heights represent mean values; brackets indicate ± SE. Numbers at base of bars indicate the numbers of rats studied in each group. Three to four SMA segments were studied in each rat.

itor of PKC activity (24). To our knowledge, this is the first in vivo study to demonstrate that inhibition of PKC activity reduces the expression of the endothelial cell adhesion molecules P-selectin and ICAM-1 induced by elevated ambient glucose. Adherence of isolated neutrophils to SMA endothelium exposed to elevated ambient glucose was blocked to a comparable degree by either SOD enzyme or bisindolylmaneimide-I. In addition, inhibition of PKC activity significantly increased release of NO from the vascular endothelium. These data strongly support the contention that inhibition of PKC activity downregulates inflammatory leukocyte-endothelium interactions by attenuation of oxidative stress and preservation of NO levels in the vascular endothelium during hyperglycemia.

Increased PKC activity has been associated with several aspects of diabetic vascular pathology such as basement membrane thickening (25), increased vascular permeability (26), and enhanced angiogenesis (27). However, very little is known about the role exerted by PKC in the regulation of leukocyte-endothelium interaction and expression of endothelial cell adhesion molecules in response to hyperglycemia in vivo.

Purified PKC has been shown to phosphorylate NO synthase (NOS) in vitro, leading to a decrease in NO production in cultured endothelial cells (28). Conversely, increased PKC activity associated with increased oxidative stress has been described in cultured endothelial cells exposed to 30 mmol/l D-glucose (29). More recent in vivo studies have reported uncoupling of NOS in the vascular endothelium of diabetic rats, leading to decreased NO release and paradoxical increased production of superoxide by the endothelial NOS (10). PKC inhibitors were able to prevent NOS uncoupling in the same animal model of diabetes and to preserve endothelial function (10) Thus, increased PKC activity appears to be responsible for decreased NO production and increased oxidative stress both in hyperglycemia (6,21) and in experimental diabetes (10). Accordingly, antioxidants such as vitamins C and E have been shown to alleviate endothelial dysfunction in several experimental animal models of diabetes and in diabetic patients (30).

Physiologic concentrations of NO exert anti-inflammatory actions by modulating expression of adhesion molecules on the vascular endothelium and by inhibiting

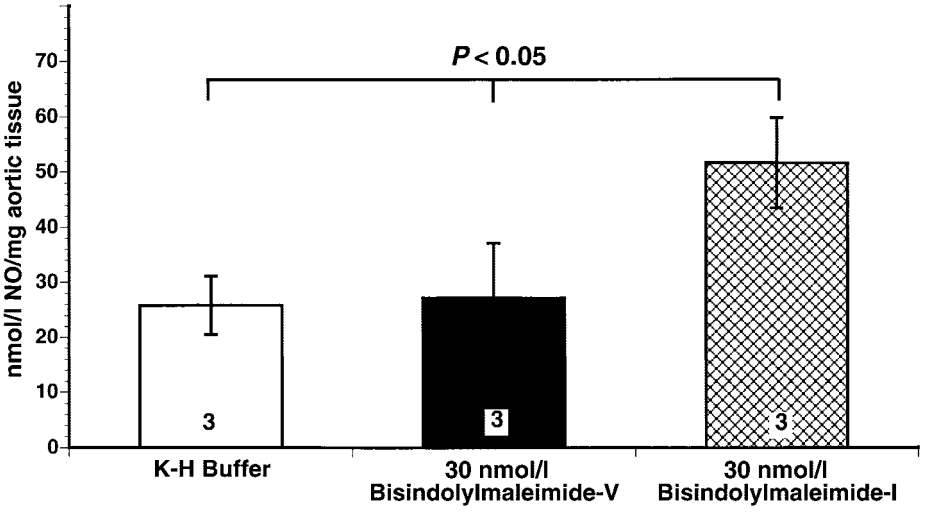

FIG. 6. Inhibition of PKC activity increases release of NO from the endothelium of the rat aorta. Incubation of isolated rat aortic strips with the PKC inhibitor bisindolylmaleimide-I significantly increased basal release of NO. Bar heights represent mean values; brackets indicate ± SE. Numbers at base of bars indicate the numbers of rats studied in each group. Three to four aortic segments were studied in each rat.

leukocyte-endothelium interactions (31). During endothelial dysfunction, reduced NO release leads to upregulation of cell adhesion molecules in the vascular endothelium (31). Under these conditions, circulating leukocytes adhere to the vascular endothelium, become activated, and release cytotoxic mediators able to cause vascular injury and tissue damage. In this regard, we have previously demonstrated that exposure of the vascular endothelium to elevated ambient glucose increases leukocyte-endothelium interaction and impairs release of endothelial NO (15).

We show evidence that inhibition of PKC activity increases release of NO from the vascular endothelium and attenuates expression of the cell adhesion molecules P-selectin and ICAM-1 in the venular endothelium of the rat mesentery exposed to elevated ambient glucose. This may represent an important mechanism by which PKC inhibitors suppress the inflammatory response induced by hyperglycemia and diabetes.

P-selectin, a glycoprotein stored in the submembranous space of Weibel-Palade bodies, can be translocated to the cell surface upon stimulation by various inflammatory mediators, including histamine, thrombin, and oxygen-derived free radicals (18,32). Once expressed on the endothelial cell surface, P-selectin promotes leukocyte rolling, an essential step in leukocyte-endothelium interaction. In this regard, we have previously shown that increased expression of P-selectin on the vascular endothelium occurs in vivo in response to elevated ambient glucose, and that activation of PKC induces translocation of P-selectin to the platelet cell membrane, a process inhibited by the NO donor, CAS1609 (33).

The endothelial cell adhesion molecule ICAM-1 is a member of the immunoglobulin family that binds the β_2 integrin CD11/CD18 expressed on the leukocyte surface. Together, these cell adhesion molecules mediate firm attachment of circulating leukocytes to the vascular endothelium, a critical, early step in the induction of inflammatory states (31). High glucose levels have been shown to increase the expression of ICAM-1 both in vitro (14) and in vivo (15).

Elevated levels of glucose increase intracellular oxidative stress in endothelial cells and upregulate PKC activity (29). Interestingly, we found evidence that under hyperglycemic conditions inhibition of PKC activity attenuates the adhesion of isolated neutrophils to rat SMA segments and that the degree of inhibition of neutrophil adhesion is comparable to that observed with SOD, a specific scavenger of superoxide radicals. Moreover, inhibition of PKC activity in the isolated rat aorta significantly increases the release of NO from the vascular endothelium. Taken together, these data provide further evidence that PKC inhibition reduces leukocyte-endothelium interactions in hyperglycemia by attenuating oxidative stress and preserving release of endothelial NO.

In summary, our data demonstrate that inhibition of PKC activity ameliorates endothelial dysfunction in hyperglycemia by attenuating leukocyte-endothelium interactions in vivo via reduced oxidative stress. The mechanism of this anti-inflammatory action of PKC inhibition is associated with inhibition of the expression of proinflammatory cell adhesion molecules on the vascular endothelium. Our data describe a novel mechanism by which increased PKC activity in response to hyperglycemia may contribute to the pathophysiology of diabetic angiopathy.

ACKNOWLEDGMENTS

G.B. and T.J.S. were supported by National Institutes of Health Training Grant no. 5T32HL07599. This work was supported by Juvenile Diabetes Research Foundation International Grant 1-200-68 to R.S.

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