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**Research Article**

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# Hypoxia-induced Exocytosis of Endothelial Cell Weibel-Palade Bodies

## A Mechanism for Rapid Neutrophil Recruitment after Cardiac Preservation

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## Abstract

**The period of hypoxia is an important priming event for the vascular dysfunction that accompanies reperfusion, with endothelial cells (ECs) and neutrophils (PMNs) playing a central role. We hypothesized that EC Weibel-Palade (WP) body exocytosis during the hypoxic/ischemic period during organ preservation permits brisk PMN recruitment into postischemic tissue, a process further amplified in an oxidant-rich milieu. Exposure of human umbilical vein ECs to a hypoxic environment ($pO_2 \approx 20$ torr) stimulated release of von Willebrand factor (vWF), stored in EC WP bodies, as well as increased expression of the WP body–derived PMN adhesion molecule P-selectin at the EC surface. Increased binding of $^{111}$In-labeled PMNs to hypoxic EC monolayers (compared with normoxic controls) was blocked with a blocking antibody to P-selectin, but was not affected by a nonblocking control antibody. Although increased P-selectin expression and vWF release were also noted during reoxygenation, hypoxia alone (even in the presence of antioxidants) was sufficient to increase WP body exocytosis. To determine the relevance of these observations to hypothermic cardiac preservation, during which the $pO_2$ within the cardiac vasculature declines to similarly low levels, experiments were performed in a rodent (rat and mouse) cardiac preservation/transplantation model. Immunodepletion of recipient PMNs or administration of a blocking anti–P-selectin antibody before transplantation resulted in reduced graft neutrophil infiltration and improved graft survival, compared with identically preserved hearts transplanted into control recipients. To establish the important role of endothelial P-selectin expression on the donor vasculature, murine cardiac transplants were performed using homozygous P-selectin deficient and wild-type control donor hearts flushed free of blood/platelets before preservation/transplantation. P-selectin–null hearts transplanted into wild-type recipients demonstrated a marked (13-fold) reduction in graft neutrophil infiltration and increased graft survival compared with wild-type hearts transplanted into wild-type recipients. To determine whether coronary endothelial WP exocytosis may occur during cardiac preservation in humans, the release of vWF into the coronary sinus (CS) was measured in 32 patients during open heart surgery. CS samples obtained at the start and conclusion of the ischemic period demonstrated an increase in CS vWF antigen (by ELISA) consisting of predominantly high molecular weight multimers (by immunoelectrophoresis). These data suggest that EC WP exocytosis occurs during hypothermic cardiac preservation, priming the vasculature to recruit PMNs rapidly during reperfusion. (*J. Clin. Invest.* 1996. 97:493–500.)**

**Key words: hypoxia • ischemia • Weibel-Palade body • neutrophils • P-selectin • von Willebrand factor**

## Introduction

Endothelial cells (EC)[1] adapt to hypoxia with a characteristic repertoire of responses (1), ranging from increased expression of endothelin (2) to increased synthesis of basic fibroblast growth factor (3). Recent studies have indicated that many features of the EC response to hypoxia parallel features of the inflammatory response; hypoxia selectively upregulates EC expression of IL-1 (4), -6 (5), and -8 (6); platelet-activating factor (PAF) (7, 8), and intercellular adhesion molecule 1 (4), which serve to fuel neutrophil (PMN) recruitment, adhesion, and activation at ischemic loci. Although these mechanisms may explain the later phases of reperfusion injury, the rapidity with which PMNs are recruited to reperfused myocardium after a period of hypothermic preservation suggests that mechanisms are involved that do not require de novo protein synthesis. In this regard, P-selectin may figure prominently in the earliest phases of PMN adhesion to the reperfused vasculature, as ECs may rapidly express preformed P-selectin from subplasmalemmal storage sites in Weibel-Palade body (WP) (9) membranes in response to the abundant oxygen-free radicals generated in the reperfusion milieu (10–12). Furthermore, recent data have pointed to a role for P-selectin–mediated leukocyte arrest in leukostasis and tissue damage associated with lung injury (13) and cardiac ischemia (14). Taken together, these findings led us to hypothesize that the hypoxic/ischemic period associated with hypothermic myocardial preservation primes the vasculature for its characteristic response during reperfusion by displaying P-selectin prominently at the EC surface before reperfusion, serving as a spark that ignites and amplifies the subsequent inflammatory response.

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1. *Abbreviations used in this paper:* CS, coronary sinus; EC, endothelial cell; HUVEC, human umbilical vein EC; LR, lactated Ringer's solution; PAF, platelet-activating factor; WP, Weibel-Palade body; vWF, von Willebrand factor.

Our experiments were designed to establish whether hypoxia per se (or hypothermic cardiac preservation, as occurs during cardiac surgery, in which the $pO_2$ within the coronary bed declines to $pO_2 < 20$ torr) (15) would result in WP body exocytosis. Furthermore, we wished to determine the role of P-selectin–dependent PMN adhesion in the cardiac graft failure that characteristically follows a period of prolonged hypothermic preservation. Our results show that hypoxia is sufficient to induce EC WP body exocytosis, even in the absence of reoxygenation (and presence of antioxidants), and that the resulting P-selectin expression causes ECs to bind PMNs in vitro. In rodents, the adverse consequences of P-selectin expression after hypothermic cardiac preservation can be completely abrogated either by neutrophil depletion, by P-selectin blockade, or by transplanting hearts whose ECs fail to express P-selectin. Because WP body exocytosis also occurs in patients undergoing open heart surgery during the period of hypothermic cardiac preservation, these data suggest that P-selectin blockade may represent a target for pharmacological intervention to improve cardiac preservation in humans.

## Methods

### *EC culture and exposure of cells to hypoxia or hypoxia/reoxygenation*

Human umbilical vein ECs (HUVECs) were prepared from umbilical cords and grown in culture by the method of Jaffe (16) as modified by Thornton (17). Experiments used confluent ECs (passages 1–4) grown in medium 199 supplemented with FBS (15%; Gemini, Bioproducts, Inc., Calabasas, CA), human serum (5%; Gemini Bioproducts, Inc.), endothelial growth supplement (Sigma Chemical Co., St. Louis, MO), heparin (90 μg/ml; Sigma Chemical Co.), and antibiotics, as described (17). When ECs achieved confluence, experiments were performed by placing cultures in an environmental chamber (Coy Laboratory Products, Ann Arbor, MI), which provided a controlled temperature (37°C) and atmosphere with the indicated amount of oxygen and carbon dioxide (5%) and the balance made up of nitrogen. Use of this chamber for cell culture experiments has been described previously (15, 18). During exposure of ECs to hypoxia (for a maximum of 16 h), the oxygen tension in the culture medium was 14–18 torr, and there was no change in the medium pH. Reoxygenation was performed by placing ECs in an ambient atmosphere containing carbon dioxide (5%) at 37°C.

### *Measurement of WP exocytosis*

ECs were plated into 24-well plates, rinsed three times with HBSS, and then exposed to hypoxia or to normoxia for the indicated durations. For experiments in which von Willebrand factor (vWF) was measured, cells were maintained in serum-free medium. All other EC experiments were performed in the EC growth medium described above. For measurement of vWF, 200-μL aliquots of culture supernatant were removed at the indicated times, and a commercially available ELISA (American Diagnostica, Inc., Greenwich, CT), based on a polyclonal goat anti–human vWF antibody, was performed on duplicate specimens, with a standard curve generated using purified human vWF antigen supplied by the same vendor. EC P-selectin expression was determined by measuring the specific binding of a murine monoclonal anti–human P-selectin antibody (WAPS 12.2 clone; Endogen, Inc., Cambridge, MA; this is an IgG1 that recognizes a calcium-sensitive epitope and blocks P-selectin–dependent neutrophil adhesion [Wicher, J., personal communication]). Antibody was radiolabeled with $^{125}I$ by the lactoperoxidase method (19) using Enzymobeads (Bio-Rad Laboratories, Hercules, CA), stored at 4°C and used within 1 wk of labeling. Binding assays were performed on HUVECs plated on 96-well plates, in which fresh M199 with 0.1% BSA (Sigma Chemical Co., St. Louis, MO) was added immediately before each experiment. Cells were placed in a humidified environment at 37°C and exposed to normoxia or hypoxia (in the presence or absence of 50 μM probucol, as indicated; Sigma Chemical Co.) for the indicated durations. Cell monolayers were fixed for 15 min with 1% paraformaldehyde[10] (cells exposed to hypoxia were fixed while still within the hypoxic environment), visually inspected to ensure that the monolayers remained intact, and washed twice with HBSS containing 0.5% BSA. Monolayers were then exposed to $10^5$ cpm of $^{125}I$-labeled anti–P-selectin antibody (WAPS 12.2) in the presence of 200 μg/mL of either unlabeled blocking antibody (WAPS 12.2) or nonblocking anti–P-selectin IgG of the same isotype (anti–GMP-140, AC1.2 clone; Becton Dickinson & Co., San Jose, CA) (20, 21). After binding for 1 h at 37°C, monolayers were washed four times with HBSS containing 0.5% BSA, and bound antibody was eluted with 1% Triton X-100 in PBS (200 μL/well) and counted. For certain experiments, cycloheximide (10 μg/mL; Sigma Chemical Co.) was added at the start of the 4-h normoxic or hypoxic period, as indicated. In separate experiments, designed to determine the degree of inhibition of protein synthesis by cycloheximide treatment, ECs were incubated with methionine- and cysteine-poor minimal essential medium (GIBCO BRL, Gaithersburg, MD) in the presence of [$^{35}S$]-methionine and [$^{35}S$]-cysteine (either in the presence or absence of cycloheximide, 10 μg/mL) (3). After 4 h of normoxic exposure, trichloroacetic acid–precipitable material was collected and counted.

### *Preparation of human PMNs and measurement of binding*

In brief, citrated blood from healthy donors was diluted 1:1 with NaCl (0.9%) followed by gradient ultracentrifugation on Ficoll-Hypaque (Pharmacia Biotech, Inc., Piscataway, NJ). After hypotonic lysis of residual erythrocytes (20-s exposure to distilled $H_2O$ followed by reconstitution with 1.8% NaCl), PMNs were suspended in HBSS with 5 mg/mL of HSA (HBSS/HSA). 50–200 $\times$ $10^6$ PMNs were suspended in HBSS/HSA in the presence of 0.2–0.5 μCi of $^{111}In$-oxine (Amersham Mediphysics, Port Washington, NY) for 15 min at 37°C. After washing with HBSS/HSA, PMNs were gently pelleted (450 g), and resuspended in HBSS/HSA to a final concentration of 5.5 $\times$ $10^6$ PMNs/mL. After gentle agitation, 100 μL of the radiolabeled PMN suspension was added to each well at the indicated time, incubated for 30 min at 37°C, and then washed four times with HBSS/HSA. Monolayers were then treated with 1 N NaOH, and the contents of each well were withdrawn and counted.

### *Heterotopic rat and mouse cardiac transplant model*

Cardiac transplants were performed using the Ono-Lindsey heterotopic isograft model of cardiac transplantation (15, 18, 22). Briefly, male Lewis rats (250–300 g, Harlan Sprague Dawley, Inc., Indianapolis, IN) were anesthetized and heparinized, and the donor heart was rapidly harvested after hypothermic high potassium cardioplegic arrest. Hearts were preserved by flushing the coronary arteries with 4°C lactated Ringer's solution (LR) (Baxter, Edison, NJ) and by 16 h of immersion in the same solution at 4°C, followed by heterotopic transplantation into gender/strain-matched recipients, with sequential donor and recipient aortic and donor pulmonary arterial/recipient inferior vena caval anastomoses performed. Graft survival was assessed by the presence/absence of cardiac electrical/mechanical activity exactly 10 min after reestablishment of blood flow, after which the graft was excised and neutrophil infiltration was quantified by myeloperoxidase activity, measured as previously described (15, 18). For certain experiments, neutrophil depletion of recipient rats was accomplished by administering a polyclonal rabbit anti–rat neutrophil antibody (23–25) (Accurate Chemical & Scientific Corp., Westbury, NY) as a single intravenous injection 24 h before the transplantation procedure. Neutrophil depletion in these animals was confirmed and quantified by counting remaining neutrophils, identified on Wright-Giemsa–stained smears of peripheral blood. In other experiments, a blocking anti–P-selectin IgG (PB1.3, 250 μg/rat; Cytel, San Diego, CA) (13, 14, 26) was administered intravenously 10 min before the onset of reperfusion. Murine heart transplants were performed in an

identical fashion using homozygous P-selectin–null or wild-type control male mice with a C57BL/6J background (27), with the harvested hearts immediately flushed free of native blood with 1.0 mL of 4°C LR administered down a cross-clamped aortic root followed by a period of hypothermic preservation consisting of 3 h of immersion in LR at 4°C.

### *Measurement of vWF in human coronary sinus effluent*

*Human coronary sinus (CS) samples.* After informed consent was obtained, CS blood was obtained at the start and conclusion of routine cardiac surgery in an unselected series of 32 patients, with simultaneous sampling of peripheral (arterial) blood in six. CS samples were obtained from a retrograde perfusion catheter, which was routinely placed in patients undergoing cardiopulmonary bypass. Plasma samples were centrifuged for 5 min at 1,500 *g* to sediment cellular elements, and the plasma was aliquoted and frozen at −70°C until the time of assay. ELISAs were performed for vWF (as described above) and thrombomodulin (Asserchron Thrombomodulin, American Bioproducts, Parsippany, NJ).

*vWF immunoelectrophoresis.* Multimeric composition of the vWF in CS plasma samples and EC supernatants was evaluated by performing agarose gel immunoelectrophoresis. Samples were diluted 1:10, 1:20, and 1:30 (as indicated) and incubated for 30 min at 37°C in native sample buffer (Bio-Rad Laboratories). Samples (20 μL) were then electrophoresed in a 1.5% agarose gel (0.675 g low $M_r$ agarose [Bio-Rad Laboratories]; 0.045 g SDS; 45 mL Tris-tricine SDS buffer [Bio-Rad Laboratories]). Molecular weight markers run simultaneously on agarose gels were visualized by marking and dividing the gel, with molecular weight marker locations assigned by Coomassie blue staining. The remaining half of the gel was washed in sodium borate (0.01 M) for 30 min followed by overnight electrophoretic transfer to a nitrocellulose membrane. The membrane was washed with washing buffer consisting of Tris-buffered saline (pH 7.5) with 0.05% Tween-20, and then blocked for 1 h with 50 mL of washing buffer containing 2.5 g of Carnation nonfat dry milk (Nestle Food Co., Glendale, CA). After rinsing with physiologic saline, the membrane was immersed overnight in washing buffer containing 1 g/dL gelatin and a 1:500 dilution of rabbit anti–human vWF serum (American Bioproducts). After washing five times with washing buffer, the membrane was immersed for 3 h with gentle shaking in washing buffer containing 1 g/dL gelatin and 16.6 μL of goat anti–rabbit horseradish peroxidase–conjugated IgG (Bio-Rad Laboratories), and developed with 60 mL of horseradish peroxidase developer (30 mg horseradish peroxidase developer powder [Bio-Rad Laboratories], 10 mL methanol, 50 mL Tris-buffered saline, and 50 μL of 30% hydrogen peroxide added just before use).

### *Statistics*

Analysis of variance was used to compare three or more conditions, with post-hoc comparisons tested using Tukey's procedure. Graft survival data was analyzed using contingency analysis with the chi-square statistic. Paired comparison of serial measurements (human CS and peripheral blood samples at the start and conclusion of cardiac surgery) were compared using Student's *t*-test for paired variables. Values are expressed as means±SEM, with a $P < 0.05$ considered statistically significant.

## Results

*Exposure of cultured ECs to hypoxia results in the release of vWF and translocation of P-selectin to the cell surface.* Previous studies have shown that exposure of ECs to hypoxia results in an elevation in intracellular calcium (28). In view of the association of increased cytosolic calcium with EC WP exocytosis in response to thrombin or histamine (29, 30), we considered whether exposure of ECs to hypoxia could initiate this process. ECs placed in an hypoxic environment ($pO_2$ 20 torr) released more vWF into the culture supernatants than their normoxic counterparts (Fig. 1 *A*, ELISA; confirmed by immunoelectrophoresis, data not shown). Although a trend towards enhanced levels of vWF was first noted by 1 h of hypoxia, the differences between normoxic and hypoxic vWF levels did not become statistically significant until 4 h of exposure, increasing steadily thereafter for up to 12 h of observation. To determine whether the increased vWF release seen by 4 h of hypoxia was due to release of preformed vWF, similar experiments were performed in the presence of 10 μg/ml cycloheximide to inhibit protein synthesis. These experiments showed that addition of cycloheximide at the start of the hypoxic period decreased hypoxia-induced vWF release by 12.5%, suggesting that the majority of vWF released by hypoxic exposure was preformed.

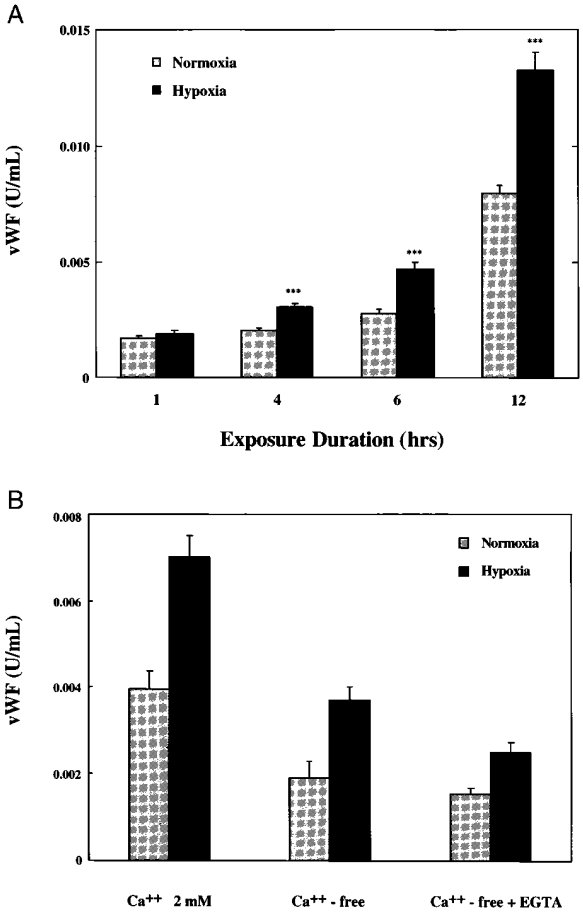


*Figure 1.* Effect of hypoxia on WP exocytosis. (*A*) Human umbilical veins were exposed to hypoxia ($pO_2$ 15–20 torr) or normoxia for the indicated durations, and vWF secretion was quantified by ELISA. ***$P < 0.001$ for hypoxia vs normoxia. (*B*) Similar experiments were performed for 8 h in the presence of 2 mM $Ca^{2+}$ (*$Ca^{++}$ 2 mM*), 0 mM $Ca^{2+}$ (*$Ca^{++}$-free*), or 0 mM $Ca^{2+}$ with 2 mM EGTA added to chelate residual extracellular $Ca^{2+}$ (*$Ca^{++}$-free + EGTA*).

Although these experiments were done in their entirety within the hypoxic environment (i.e., there was no reoxygenation), to further demonstrate that this hypoxia-mediated exocytosis of WPs was independent of the formation of reactive oxygen intermediates, the antioxidant probucol (50 μM) was added to the ECs at the onset of hypoxia and was found to

have no effect (vWF 4.7±0.31 × $10^{-3}$ U/ml at 6 h of hypoxia). The presence of probucol blunted the further increase in vWF levels seen after reoxygenation of the hypoxic ECs (data not shown). The calcium dependence of hypoxia-induced WP exocytosis was demonstrated by experiments in which ECs were placed in a calcium-free medium at the start of hypoxic exposure. Absence of extracellular calcium attenuated hypoxia-induced EC release of vWF, and addition of EGTA had an even more suppressive effect (basal endothelial release of vWF was also diminished by the reduction of extracellular calcium) (Fig. 1 *B*).

To determine whether hypoxia also induced translocation of P-selectin to the EC plasmalemmal surface, specific binding of $^{125}$I-labeled anti–P-selectin IgG to normoxic or hypoxic EC monolayers was examined. Binding studies were performed on EC monolayers fixed with paraformaldehyde while still within the hypoxic environment, to obviate oxygen-free radical–induced P-selectin expression during reoxygenation. These studies demonstrated enhanced binding of $^{125}$I–anti–P-selectin IgG by hypoxic compared with normoxic ECs (Fig. 2 *A*). This binding was blocked by unlabeled anti–P-selectin IgG (WAPS 12.2 clone). Surface expression of P-selectin was noted at the earliest time points observed (60 min of hypoxia) and was observed at similar levels throughout the period of hypoxic exposure (up to 4 h of observation). It is possible that hypoxia-induced endothelial P-selectin expression was detected at time points preceding a statistically significant increase of vWF release in similarly treated cells, because a portion of the initially secreted vWF binds tightly to subendothelial matrix (31).

To determine whether protein synthesis was required for hypoxia-induced P-selectin expression, a separate experiment was performed in which cycloheximide was given at the onset of normoxia or hypoxia, and binding of radiolabeled anti–P-selectin IgG determined at the 4-h time point. This experiment demonstrated that even with > 85% inhibition of protein synthesis (Fig. 2 *B*, *inset*), hypoxia still increased endothelial P-selectin expression, albeit at reduced levels (Fig. 2 *B*). To establish that hypoxia-induced cell-surface P-selectin may participate in neutrophil binding, human neutrophils radiolabeled with $^{111}$In-oxine were incubated with hypoxic ECs; enhanced binding to hypoxic monolayers was observed. Hypoxia-induced $^{111}$In-PMN binding was blocked by the addition of a blocking anti–P-selectin IgG, but not by a nonblocking anti–P-selectin IgG of the same isotype (Fig. 2 *C*).

*Role of P-selectin–dependent neutrophil adhesion in hypothermic/ischemic myocardial preservation.* To establish the relevance of these observations to hypothermic myocardial preservation (in which the $pO_2$ of the preservation solution within the coronary vasculature drops below 20 torr [15]), hearts were harvested from male Lewis rats and subjected to hypothermic

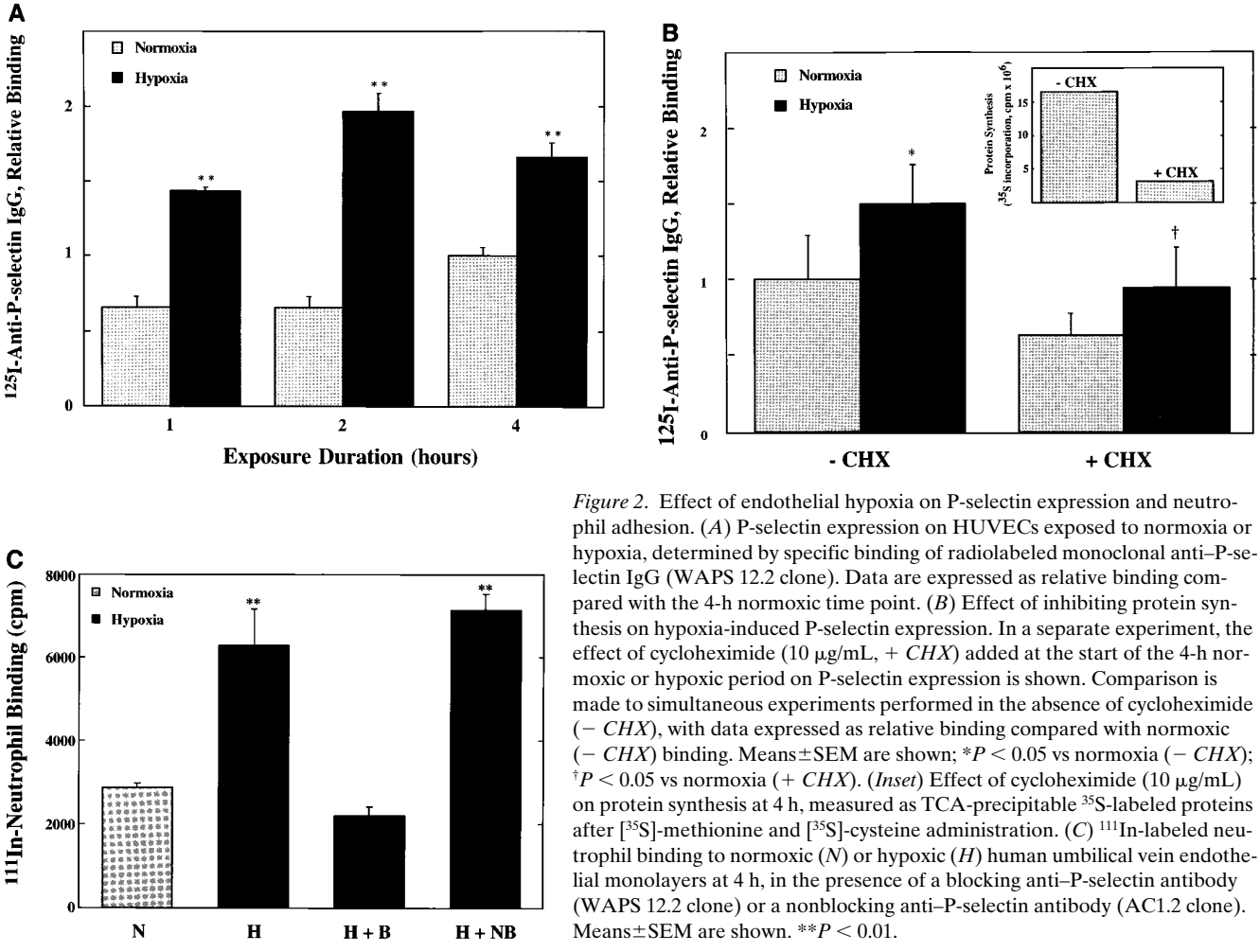


*Figure 2.* Effect of endothelial hypoxia on P-selectin expression and neutrophil adhesion. (*A*) P-selectin expression on HUVECs exposed to normoxia or hypoxia, determined by specific binding of radiolabeled monoclonal anti–P-selectin IgG (WAPS 12.2 clone). Data are expressed as relative binding compared with the 4-h normoxic time point. (*B*) Effect of inhibiting protein synthesis on hypoxia-induced P-selectin expression. In a separate experiment, the effect of cycloheximide (10 μg/mL, + *CHX*) added at the start of the 4-h normoxic or hypoxic period on P-selectin expression is shown. Comparison is made to simultaneous experiments performed in the absence of cycloheximide (− *CHX*), with data expressed as relative binding compared with normoxic (− *CHX*) binding. Means±SEM are shown; *$P < 0.05$ vs normoxia (− *CHX*); †$P < 0.05$ vs normoxia (+ *CHX*). (*Inset*) Effect of cycloheximide (10 μg/mL) on protein synthesis at 4 h, measured as TCA-precipitable $^{35}$S-labeled proteins after [$^{35}$S]-methionine and [$^{35}$S]-cysteine administration. (*C*) $^{111}$In-labeled neutrophil binding to normoxic (*N*) or hypoxic (*H*) human umbilical vein endothelial monolayers at 4 h, in the presence of a blocking anti–P-selectin antibody (WAPS 12.2 clone) or a nonblocking anti–P-selectin antibody (AC1.2 clone). Means±SEM are shown. **$P < 0.01$.

preservation as described in Methods. Because neutrophil-mediated damage after cardiac ischemia is well established (32–38), the potential pathophysiologic role of endothelial P-selectin expression was investigated in an orthotopic rat heart transplant model in which reperfusion occurred after a period of hypothermic preservation. These experiments showed excellent graft survival and little neutrophil infiltration if heart transplantation was performed immediately after harvest (Fig. 3 *A*, *Fresh*). However, when similar experiments were performed with an intervening (16-h) period of hypothermic preservation between the harvest and transplantation procedures, there was a high incidence (90%) of graft failure and marked leukostasis, confirmed histologically (not shown) and by determining myeloperoxidase activity (Fig. 3 *A*, *Prsvd*). To demonstrate that neutrophil adhesion was responsible, at least in part, for graft failure after prolonged preservation, transplants were performed after neutrophil depletion of recipient rats. The polyclonal rabbit anti–rat PMN antibody that we used (23–25) eliminated virtually all circulating PMNs in the recipients (PMN count 1,471±56 vs 67±11 PMN/mm$^3$ for control and immunodepleted animals, respectively, $P < 0.001$), with little effect on other cell types. When 16-h preserved hearts were transplanted into neutrophil-depleted recipients to provide a neutrophil-free reperfusion milieu, there was a significant reduction in graft myeloperoxidase activity and an increase in graft survival (Fig. 3 *A*, *Prsvd (−) PMN*). Normal recipient rats infused with blocking anti–P-selectin IgG (PB1.3) 10 min before reestablishment of blood flow demonstrated a reduction of both myeloperoxidase activity as well as improvement in graft survival (Fig. 3 *A,* α *PS, Blocking*) of a similar magnitude as neutrophil-depleted recipients. This reduced PMN infiltration and improved graft survival was observed despite 16 h of hypothermic preservation of the donor heart. In sharp contrast, administration of a nonblocking control antibody (AC1.2) had no beneficial effect on graft leukostasis or graft survival (Fig. 3 *A,* α-*PS, Non-blocking*).

Because in addition to the interactions between ECs and PMNs, platelets may also interact with PMNs via a P-selectin–dependent mechanism (39), we designed an experiment to isolate the contribution of endothelial P-selectin to the leukostasis and graft failure that occur after prolonged hypothermic cardiac preservation. For these experiments, donor hearts from homozygous P-selectin–deficient mice could be flushed free of blood, so that P-selectin–null coronary ECs could be transplanted into wild-type recipients with P-selectin–containing platelets. Using a murine heterotopic heart transplant model performed identically to the rat operation, donor hearts were obtained from either homozygous P-selectin–null mice (27) or wild-type controls; all hearts were transplanted into wild-type recipients. These experiments demonstrated a significantly higher graft survival rate in the P-selectin–null → wild-type transplants compared with wild-type → wild-type transplants (Fig. 3 *B*). This improved graft survival in the former group was paralleled by a marked (13-fold) reduction in graft leukostasis (Fig. 3 *B*). Because these hearts had been flushed free of blood at the start of preservation, these studies implicate coronary endothelial (rather than platelet-derived) P-selectin in the poor preservation and leukocyte arrest noted after hypothermic myocardial preservation.

*WP exocytosis during human cardiac surgery.* To establish the relevance of these findings to humans, the next set of experiments was designed to demonstrate that coronary ECs release the contents of WPs during hypothermic cardiac preservation as occurs during routine cardiac surgery. Measurements were made of vWF release from the coronary vasculature during a well-defined period of cardiac ischemia, which occurs during the period of aortic cross-clamping. CS blood was sampled at the start ($CS_1$) and conclusion ($CS_2$) of aortic cross-clamping in 32 patients (this interval represents the ischemic period). These patients (23 male, 9 female) had a clinical history of valvular heart disease ($n = 11$) or ischemic heart disease ($n = 21$), and underwent either valve repair/replacement or coronary artery bypass grafting, respectively. Capture ELISAs performed for the integral membrane protein thrombomodulin (40) demonstrated no change in levels between the $CS_1$ and the $CS_2$ samples (4.35±1.2 ng/mL vs 3.48±0.8 ng/mL, $P$ = NS), suggesting that ECs were not sloughed and cell mem-

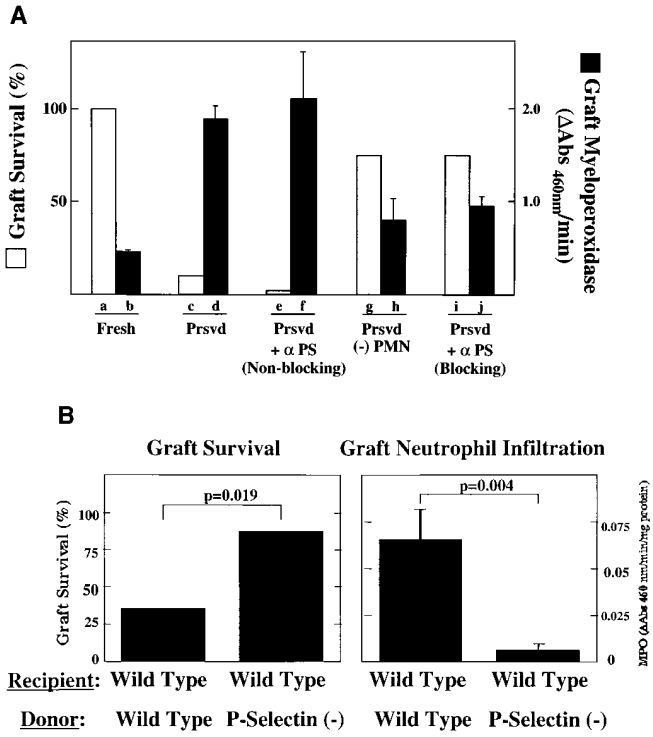


*Figure 3.* Role of neutrophils and endothelial P-selectin in rodent cardiac preservation followed by heterotopic transplantation. (*A*) Rat cardiac preservation. Hearts were transplanted immediately after harvest (*Fresh, n* = 8) or preserved for 16 h in LR at 4°C followed by transplantation (*Prsvd, n* = 4). The effect of administering nonblocking anti–P-selectin antibody (AC1.2, *n* = 3), immunodepleting recipients of neutrophils before donor heart implantation (− *PMN, n* = 4), or administering 250 μg of blocking anti–P-selectin IgG (PB1.3, *n* = 4) 10 min before reperfusion on cardiac graft survival (*shaded bars*) and leukostasis (myeloperoxidase activity, *solid bars*). Means±SEM are shown. For graft survival, *c* vs *a*, $P < 0.0001$; *g* vs *c*, $P < 0.05$; *i* vs *e* or *c*, $P < 0.05$. For graft neutrophil infiltration, *d* vs *b*, $P < 0.01$; *h* vs *d*, $P < 0.05$; *j* vs *d* or *f*, $P < 0.05$. (*B*) Role of coronary endothelial P-selectin in cardiac preservation, using donor hearts from P-selectin–null (or wild-type control) mice that were flushed free of blood before preservation. (*B, left*) Graft survival was assessed by the presence/absence of cardiac electrical/mechanical activity exactly 10 min after reestablishment of blood flow. (*B, right*) Quantification of neutrophil infiltration by measurement of myeloperoxidase activity (dABs 460 nm/min) as described (15, 18). (For bars shown from left to right, *n* = 14, 8, 13, and 7, respectively, with *P* values indicated).

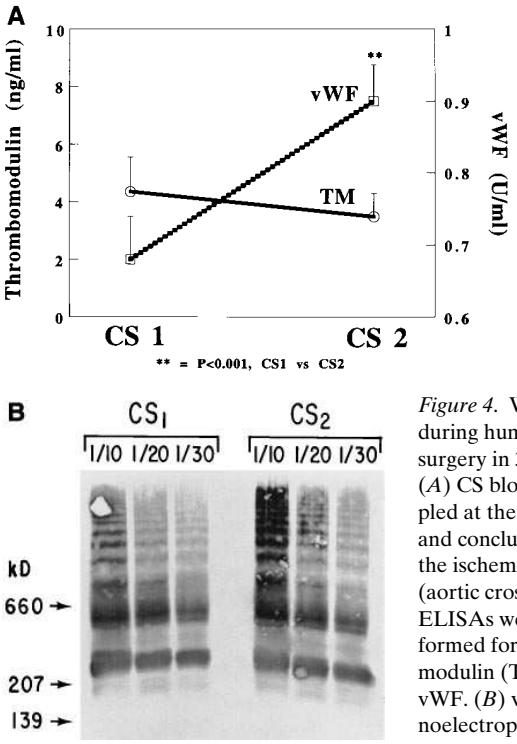


*Figure 4.* WP release during human cardiac surgery in 32 patients. (*A*) CS blood was sampled at the start ($CS_1$) and conclusion ($CS_2$) of the ischemic period (aortic cross-clamping). ELISAs were performed for thrombomodulin (TM) and vWF. (*B*) vWF immunoelectrophoresis of a representative sample of $CS_1$ and $CS_2$ blood from the same patient (dilution factors are indicated). There is an increase in high molecular weight multimers detected in the $CS_2$ samples.

brane integrity was maintained during cardiac preservation. Similar measurements performed for vWF showed that there was a consistent and significant increase in vWF that is secreted during the course of cardiac preservation (0.68±0.06 U/ml vs 0.90±0.05 U/ml, $CS_1$ vs $CS_2$, $P < 0.01$) (Fig. 4 *A*).

To demonstrate that this vWF was likely to be of coronary endothelial rather than of platelet origin, and hence was not simply a consequence of cardiopulmonary bypass, peripheral blood samples were obtained simultaneously with the $CS_1$ and $CS_2$ samples and showed that levels of vWF were unchanged (0.813±0.52 U/mL vs 0.900±0.41 U/mL, $P$ = NS), suggesting that mechanical perturbation of platelets during cardiopulmonary bypass was not causative. Because vWF is present in plasma as multimers with a range of $M_r$s (41–44), with those vWF multimers from the stimulatable pool (as opposed to those constitutively secreted) being of the highest molecular weight (45), immunoelectrophoresis was performed on the CS samples. These gels demonstrated that, in addition to an overall increase in vWF in the $CS_2$ samples, there appeared to be an increase in high molecular weight multimers, suggesting release from a stimulatable pool, as is found in ECs (Fig. 4 *B*).

## Discussion

The vasculature plays a critical role in maintaining the extracellular milieu of organs subjected to ischemia and reperfusion, a role that is chiefly orchestrated by the ECs lining the endovascular lumen. The EC responds to a period of oxygen deprivation by striking phenotypic modulation, becoming prothrombotic (46) and proinflammatory (1, 4, 6). ECs exposed to hypoxia secrete the proinflammatory cytokines IL-1 (4) and IL-8 (6), which may serve to direct leukocyte traffic to areas of ischemia. Because these processes require de novo protein synthesis, they do not explain the immediate events that occur after a period of hypothermic preservation. Whereas enhanced expression of intercellular adhesion molecule 1 and induction of E-selectin may contribute at later times to leukocyte arrest in cardiac grafts, this does not explain the rapid leukostasis observed after cold preservation, in which protein synthesis is likely to be considerably slowed. In this context, cycloheximide pretreatment does not alter the early (90–120-min) PMN adhesion seen after hypoxic exposure of ECs (7), suggesting that de novo protein synthesis need not be involved in hypoxia-mediated increases in PMN binding. Although PAF may participate in hypoxia-mediated PMN adhesion (7, 47) and activation (48, 49), PAF is not stored and must be synthesized, which may lessen its importance during the hypothermic period during myocardial preservation. It is for this reason that rapid EC expression of preformed P-selectin from subplasmalemmal storage sites in WPs (9, 50, 51) may represent the most important mechanism for early PMN recruitment after hypothermic preservation. WPs are found in abundance within the coronary microvasculature (52), suggesting their particular importance in cardiac preservation.

Our data show WP exocytosis occurs in response to hypoxia per se as well as in human hearts during hypothermic preservation. Whereas it is difficult to identify precisely an endothelial origin for the vWF observed in the human CS samples, studies of platelets after cardiopulmonary bypass demonstrate no increase in surface P-selectin expression or α-granule secretion (53, 54). This suggests that the observed increase in CS vWF after aortic cross-clamping is not of platelet origin. Two aspects of our data also suggest that the vWF released after ischemia is of endothelial origin: (*a*) Peripheral vWF levels remained unchanged whereas CS levels are increased after myocardial ischemia, suggesting that the elevated vWF was emanating from the heart rather than from the cardiopulmonary bypass apparatus; and (*b*) the transgenic, P-selectin–null donor hearts were flushed free of donor blood at the onset of preservation, so that when transplanted into wild-type recipients, presumably coronary endothelial (not platelet) P-selectin is absent. These experiments demonstrate the important contribution of endothelial P-selectin to the neutrophil recruitment that accompanies reperfusion.

It is not surprising that P-selectin should be important after hypothermic myocardial preservation; recent studies have demonstrated that P-selectin is an important mediator of neutrophil-induced reperfusion damage after normothermic ischemia, as has been shown in rabbit ear (26) and feline cardiac ischemia (14) models. Because oxidants cause expression of P-selectin at the EC surface (10), it was important in our studies to evaluate the role of the hypoxic period alone, as it may prime ECs to recruit the first wave of PMNs, with further PMN recruitment amplified with the onslaught of reactive oxygen intermediates produced in the reperfusion microenvironment. Although one previous report has suggested that hypoxia might induce EC P-selectin expression, these experiments (7) were actually performed after reoxygenation, a condition known to induce both superoxide (18, 55) and neutrophil adherence to cultured ECs (56). Our experiments were performed entirely within a hypoxic environment to prevent completely the possibility of reoxygenation, and antioxidants

failed to block hypoxia-induced P-selectin expression, suggesting that our observations reflect hypoxia per se rather than reoxygenation. Furthermore, the cardiac protection we have demonstrated using a strategy whereby blood-free preserved hearts from transgenic P-selectin–null mice are transplanted into recipients with wild-type platelets demonstrates that endothelial P-selectin expression can be deleterious after hypothermic cardiac preservation. Because WP exocytosis occurs during hypothermic cardiac preservation in humans, these studies suggest that myocardial preservation may be enhanced by therapeutic strategies designed to block the activity of P-selectin expressed at the endothelial surface.

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