Initial Contact and Subsequent Adhesion of Human Neutrophils or Monocytes to Human Aortic Endothelial Cells Releases an Endothelial Intracellular Calcium Store

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Background  Increases in both leukocyte and endothelial cytosolic free [Ca\(^{2+}\)] may be involved in intercellular adhesion by regulating the affinity of surface adhesion molecules or by facilitating transendothelial leukocyte migration. The purpose of this study was to examine the effect of initial contact and subsequent adhesion of human neutrophils or monocytes on human aortic endothelial [Ca\(^{2+}\)].

Methods and Results  Endothelial monolayers were loaded with the fluorescent Ca\(^{2+}\) indicator indo 1 and exposed to isolated human peripheral blood neutrophils or to a cultured human monocyte cell line. A rapid, fourfold increase in endothelial cytosolic [Ca\(^{2+}\)] occurred within seconds of leukocyte contact. No increase in endothelial [Ca\(^{2+}\)] occurred on contact of 18.25-μm inert microspheres, isolated red blood cells, or suspensions of cultured human aortic endothelial cells. In experiments performed on monolayers grown in 1-mm² capillary flow tubes, the increase in endothelial cytosolic [Ca\(^{2+}\)] on initial leukocyte contact was found to be related to the subsequent resistance to leukocyte detachment during exposure to arterial levels of shear stress (13.4 dyne·cm\(^{-2}\)). The increase in endothelial cytosolic [Ca\(^{2+}\)] during leukocyte contact was not inhibited in Ca\(^{2+}\)-free buffer but was abolished by prior depletion of an endoplasmic reticulum Ca\(^{2+}\) store by thapsigargin. Pretreatment of neutrophils with R15.7, a specific monoclonal antibody to the adhesion protein CD-18, inhibited the increase in endothelial cytosolic [Ca\(^{2+}\)] on neutrophil contact.

Conclusions  Initial contact leading to subsequent adhesion of human leukocytes to human aortic endothelial cells releases an endothelial intracellular Ca\(^{2+}\) store. This may, in part, be mediated by specific adhesion proteins and may in turn regulate the affinity of surface adhesion molecules or facilitate transendothelial migration of leukocytes. (Circulation. 1994;90:1899-1907.)

Key Words  • endothelium • leukocytes • indo 1 • shear stress

Leukocyte adhesion to the vascular endothelium is important in the processes of inflammation and postischemic reperfusion injury and in the early events of atherosclerosis. An increase in leukocyte cytosolic [Ca\(^{2+}\)], ([Ca\(^{2+}\)])\(_{i}\), appears to be involved in intercellular adhesion. Polymorphonuclear leukocytes (PMNs) exhibit an increase in [Ca\(^{2+}\)]\(_{i}\) during adherence to prestimulated endothelial cells,\(^{1}\) and adherent PMNs demonstrate multiple [Ca\(^{2+}\)]\(_{i}\) transients that may be blocked by antibodies against specific integrins.\(^{2,3}\) Transmembrane signaling in lymphocytes by the T3-antigen receptor complex involves an inositol-1,4,5-triphosphate (InsP\(_3\))--mediated increase in lymphocyte [Ca\(^{2+}\)].\(^{4}\) In monocytes, a sustained increase in [Ca\(^{2+}\)]\(_{i}\) may regulate the high-affinity receptor function of CD11b/CD18,\(^{5}\) and cross-linking of the myeloid differentiation protein CD14 on monocytes induces a transient increase in monocyte [Ca\(^{2+}\)], and enhances monocyte adhesion to prestimulated endothelial cells.\(^{6}\)

Recently, a transient increase in endothelial [Ca\(^{2+}\)] was demonstrated during adhesion and transendothelial migration of PMNs prestimulated with formyl-methionyl-leucyl-phenylalanine (fMLP).\(^{7}\) This effect on [Ca\(^{2+}\)]\(_{i}\) appeared to increase the permeability of the endothelial monolayer and to thereby regulate PMN migration but was not found to alter PMN-endothelial adhesion. In contrast, another recent study\(^{8}\) demonstrated that contact of tumor cells with bovine pulmonary artery endothelial cell monolayers caused a transient increase in [Ca\(^{2+}\)], that enhanced cell-cell adhesion.

Several lines of indirect evidence also suggest that an increase in endothelial [Ca\(^{2+}\)] might be important in leukocyte adhesion or migration. The coagulation protein thrombin increases endothelial [Ca\(^{2+}\)]\(_{i}\) and rapidly induces endothelial cells in vitro to become adhesive for PMNs\(^{9,10}\) and monocytes.\(^{11}\) Similarly, exposure of endothelial cells to hydrogen peroxide (H\(_2\)O\(_2\)) increases endothelial [Ca\(^{2+}\)]\(_{i}\), and enhances PMN adhesion.\(^{12}\) Histamine stimulation, which increases endothelial [Ca\(^{2+}\)],\(^{13,14}\) also results in the rapid translocation to the membrane of the P-selectin GMP-140, which mediates endothelial cell adhesion to PMNs. The Ca\(^{2+}\) ionophore ionomycin acts synergistically with the PKC agonist phorbol 12-myristate 13-acetate (PMA) to upregulate expression of the inter-
cellular adhesion molecule–1 (ICAM-1) on endothelial cells. In addition, transendothelial migration of leukocytes might be affected by endothelial [Ca\(^{2+}\)], since an increase in endothelial [Ca\(^{2+}\)] regulates vascular permeability by opening intercellular junctions.

No previous study has examined whether [Ca\(^{2+}\)], increases in human arterial endothelium during adhesion of nonstimulated PMNs or monocytes. In addition, the source of the increase in endothelial [Ca\(^{2+}\)], during chemotactrant-stimulated leukocyte adhesion has not been identified. We therefore studied the effect of the interaction of nonstimulated human peripheral blood PMNs and a human monocyte cell line on nonstimulated human aortic endothelial cell [Ca\(^{2+}\)]. The use of human aortic endothelial cell monolayers in this study is relevant to both postischemic reperfusion injury and to atherogenesis, as these both appear to be initiated by interactions between circulating blood cells and arterial endothelium. In this study, we also examined whether an increase in endothelial [Ca\(^{2+}\)], during the initial interaction with leukocytes was related to the subsequent ability to resist detachment despite exposure to arterial levels of hemodynamic shear stress in experiments performed in capillary flow tubes.

**Methods**

**Cell Culture**

Human aortic endothelial cells were obtained as proliferating quaternary cultures (Clonetics) and grown to confluence to passage 5-7 in endothelial cell growth medium supplemented with 2% fetal bovine serum (FBS), 50 μg/mL gentamicin, 50 ng/mL amphotericin-B, and 12 μg/mL bovine brain extract in a 37°C humidified atmosphere of 95% air–5% CO\(_2\). Bovine aortic endothelial cells (NIA Aging Cell Repository) were grown to passage 19-21 in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (GIBCO) in a 37°C humidified atmosphere of 95% air–5% CO\(_2\). Identification of endothelial cells was performed by demonstrating specific immunofluorescent staining for factor VIII-related antigen and for acetylated low-density lipoprotein (DiI-Ac-LDL). The U937 human monocytic cell line (American Type Culture Collection) was maintained in DMEM with 10% FBS in a 95% air–5% CO\(_2\) humidified atmosphere at 37°C. The characteristics of the adhesive interactions between this cell line and cultured vascular endothelial cells have been previously described.

**Use of Glass Capillaries for Graded Shear Stress Exposure Following Initial Leukocyte Contact**

For some experiments, endothelial cells were grown in boreislicate glass capillary tubes (1 mm\(^2\)×5 cm; Vitro Dynamics) as previously described to facilitate exposure of the monolayer to arterial levels of shear stress calculated by the formula: \(r = \mu \frac{Q}{\pi r^4} \) where \(\mu\) is fluid viscosity, \(Q\) is flow rate, and \(r\) is internal radius (half-width) of the tube. Exposure to hemodynamic shear stress was used to determine the resistance of leukocytes to detachment as a means of identifying firm leukocyte-endothelial adhesion and to establish the relation between adhesion and the change in endothelial [Ca\(^{2+}\)]. Following the period of leukocyte contact with the endothelial monolayer under static conditions, increasing flow rates (and shear stress) were used to detect resistance to detachment.

Capillary tubes were precoated with a solution of 1% gelatin (Sigma Chemical Co) in phosphate-buffered saline (PBS). The gelatin solution entered the tubes by capillary action and coated the glass for a minimum of 2 hours in a 37°C, 95% air–5% CO\(_2\) incubator. The tubes were washed with PBS before cell plating. Cells grown in tissue culture dishes were exposed to a solution of 0.25% trypsin and 0.5 mmol/L EGTA (Sigma) and plated at a concentration of 5×10\(^4\) cells/mL in the flow tubes. Cells formed a confluent monolayer on one face of the capillary tube after 48 hours at 37°C in a 95% air–5% CO\(_2\) humidified atmosphere. Monolayers grown in this manner exhibited contact inhibition and retained the typical “cobblestone” morphological characteristics of endothelial cells grown on tissue culture plastic. Immunofluorescent staining for factor VIII–related antigen and for DiI-Ac-LDL uptake was also performed on cells grown in capillary tubes as described above. Other experiments were performed on endothelial cells grown on gelatin-coated glass coverslips (Corning) in which exposure to shear stress to detect resistance to detachment was not used.

**PMN Preparation**

PMNs were prepared from fresh blood obtained from healthy human volunteers by the method of Kensler and Trush, yielding neutrophils with a purity of >95%. Venous blood was drawn in heparinized 10-mL Vacutainers and centrifuged at 500g in a Beckman TJ-6 for 10 minutes at room temperature. The plasma and buffy coat were decanted, and the red cell layer was mixed with an equal volume of 6% dextran [MW 500,000], 5 g dextran [MW 80,000], and 100 mL 0.9% saline. The mixture was then transferred to 3-mL plastic syringes, which were inverted and incubated at 37°C for 1 hour until clear separation was noted. The upper layer was then ejected through a 16-gauge needle with a 90° bend into a 50-mL plastic centrifuge tube and spun for 10 minutes at 500g at room temperature. The supernatant was decanted, and the pellet was resuspended in ice-cold ACK lysis buffer (0.155 mol/L NH\(_4\)Cl, 0.01 mol/L KHCO\(_3\), and 0.1 mmol/L EDTA [pH 7.4]) and centrifuged again for 10 minutes at 50g at room temperature. The pellet was then washed, resuspended in PBS with 1% glucose, and centrifuged for 5 minutes at 340g at room temperature. The final PMN suspension was counted using a hemacytometer, stored on ice in 24 mmol/L PIPES buffer with 5% human serum albumin and 5.6 mmol/L d-glucose (PAG), and used within hours of preparation.

In some experiments, PMNs were pretreated with the monoclonal antibody (Mab) to CD-18, MAb R15.7, obtained from Dr Robert Rothlein of Boehringer Ingelheim. PMNs were incubated with 40 μg/mL of R15.7 at 0°C for 30 minutes, which would be expected to saturate PMN CD-18.

**Measurement of Endothelial [Ca\(^{2+}\)]**

To assess changes in [Ca\(^{2+}\)], we loaded endothelial monolayers with the acetoxymethyl ester derivative of the fluorescent Ca\(^{2+}\)-probe indo 1 (indo 1 AM; Molecular Probes). Capillary tubes containing the endothelial monolayers were loaded with growth medium containing 12 μmol/L indo 1 AM and kept in a 95% air–5% CO\(_2\) incubator at room temperature for 60 minutes. The tubes were then washed with indicator-free buffer and kept in the incubator for an additional 60 minutes to allow more complete deesterification of indo 1 before being mounted on the stage of a modified inverted fluorescence microscope (Zeiss IM-35) where emitted indo 1 fluorescence was monitored as previously described. Briefly, fluorescence was excited at 350±5 nm using a xenon strobe lamp (model 236 strobeoscope and model 355 lamp; Chadwick-Helmuth Electronics), bandpass interference filters (Andover), and selected wavelength bands of emitted fluorescence at 391 to 434 nm (“410-nm channel”) and 457 to 507 nm (“490-nm channel”) corresponding to the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms of the indicator, respectively. The 410 nm–to–490 nm ratio obtained from an area of the tube approximately 180 μm in diameter was used as an index of [Ca\(^{2+}\)]. The fluorescence of a comparable field of unloaded endothelial cells was <5% of indo 1–loaded cells and was not significantly altered by the addition of leukocytes.

To approximate [Ca\(^{2+}\)] values from indo 1 fluorescence ratios, the intracellular minimal and maximal ratios (R\(_{\text{min}}\) and R\(_{\text{max}}\), respectively) were determined as previously described. To
determine $R_{\text{max}}$, indo 1–loaded monolayers were superfused with a solution containing (in mmol/L): NaCl 137.0, KCl 5.0, MgSO4 1.2, NaH2PO4 1.2, d-glucose 16, HEPES 10, and EGTA 2 (pH 7.40±0.01). Cells were then exposed to a solution of similar composition with 10 mmol/L EGTA and 0.05% Triton X-100, resulting in a rapid decrease in the indo 1 ratio before an appreciable loss of indicator occurred, determined by simultaneous recording of absolute fluorescence at each wavelength as well as the 410 nm–to–490 nm ratio. To determine an intracellular $R_{\text{max}}$, different monolayers were exposed to a solution containing 132 mmol/L KCl, 10 mmol/L K-HEPES, 1 mmol/L MgSO4, 2 μmol/L rotenone, 2 μmol/L carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP, Sigma), and 10 ng/mL valinomycin (Calbiochem). Cells were then exposed to a similar solution containing 2 μmol/L ionomycin, 69.2 mmol/L CaCl2, and 100 mmol/L N-hydroxyethyl-ethylenediamine-triacetic acid; this resulted in free [Ca2+] of 5900 mmol/L. In this system, the intracellular $R_{\text{max}}$ was 2.64 and $R_{\text{max}}$ was 0.40; these were used to provide approximations of [Ca2+], according to the formula [Ca2+] = $K_d$ (R–$R_{\text{max}}$–$R_{\text{max}}$ – R) ($S_2$/$S_1$),27 where $K_d$ is the effective dissociation constant of indo 1 and $S_1$ and $S_2$ are the fluorescence intensities at 490 nm of Ca2+-free and saturated dye, respectively. $K_d$ was extrapolated from the published determination using the Chelex assay by Lattanzio.28

Experimental Protocol
In the initial series of experiments, PMNs or U937 monocytes were resuspended in a buffer containing (in mmol/L): NaCl 137, KCl 5, NaH2PO4 1.2, MgSO4 1.2, d-glucose 15, HEPES 20, and CaCl2 1.5 (pH 7.40) and perfused through the capillary tube (flow=0.2 mL/min, shear stress=0.3 dyne cm−2) containing the indo 1–loaded endothelial monolayer using a unipulsatile, integrated drive positive displacement pump (Cole-Parmer) or a Harvard syringe pump (South Natick). From 5 to 10 leukocytes were added for each endothelial cell in the monolayer. After the leukocytes entered the tube, flow was stopped, and the leukocytes were allowed to settle for 10 minutes on the endothelial cell monolayer as emitted indo 1 fluorescence was continuously collected. The monolayer was simultaneously illuminated with red (650 to 750 nm) light through the normal bright-field illumination optics of the microscope, and the image was visualized through a 100× fluorophotomicroscopy objective (Nikon). The bright-field and fluorescence images were separated by a 600-nm short-pass dichroic mirror, and the bright-field image was directed to a television camera and monitor, permitting the experiments to be videotaped for subsequent analysis during simultaneous recording of indo 1 fluorescence. The endothelial monolayer was kept in the plane of focus and the time of contact between the leukocyte and the endothelial cell monolayer coincided with the time when the leukocytes entered the plane of focus. Typically during the first 2 minutes of the 10-minute period, approximately 15 to 20 leukocytes were observed to contact the endothelial monolayer. At the end of the experiment, the video recording was analyzed and matched to the indo 1 fluorescence record. In this way, the leukocyte that entered the plane of focus of the endothelial monolayer (ie, made contact with the monolayer) at the time of the rapid rise in endothelial [Ca2+] could be specifically identified. After the 10-minute period without flow, flow was increased stepwise to achieve shear stresses of 1.7, 3.4, and 6.7 dyne cm−2, and each level of shear stress was maintained for 30 seconds. They were then exposed to a maximal shear stress of 13.4 dyne cm−2 (flow rate=8 mL min−1) for 1 minute. In this way, the increase in endothelial [Ca2+] on initial leukocyte contact was able to be related to firm leukocyte adhesion, defined as the ability to resist detachment during exposure to these shear stresses.

Studies were also performed on indo 1–loaded endothelial cells cultured on glass coverslips placed in an open Lucite chamber, and fluorescence was monitored as described above. Leukocytes entered the chamber by gentle pipetting from above and were allowed to settle on the monolayer grown on a glass coverslip. The cells were visualized throughout the course of the experiment as indo 1 fluorescence was collected. It was therefore possible to relate changes in [Ca2+], to the time of leukocyte-endothelial cell interaction. Experiments were performed at 23°C since more significant and rapid intracellular loss of indo 1 occurs at 37°C than at 23°C.25

Statistical Analysis
Data are presented as the mean±SEM. Statistical comparisons between the changes in endothelial [Ca2+], initiated by contact with PMNs pretreated with MAb 15.7 and those not pretreated were analyzed using an unpaired Student’s t test. A difference was considered significant at P<.05.

Results

PMN Adhesion Increases Endothelial [Ca2+]

Human aortic endothelial cells grown in glass capillary tubes formed a characteristic monolayer that retained the typical “cobblestone” morphological appearance of endothelial cells grown on tissue culture plastic (Fig 1). During the interaction of human peripheral blood PMNs and indo 1–loaded human aortic endothelial cells, a rapid reversible fourfold to fivefold increase in [Ca2+] was observed (Fig 2) within seconds of PMN contact, returning toward control values over several minutes. The response was similar in magnitude and time course to the increase in [Ca2+], on stimulation of human aortic endothelial cells with 10 μmol/L histamine (n=4, not shown) and was observed in both bicarbonate-free buffer (HEPES, 1.5 mmol/L Ca2+, approximate increase in [Ca2+], from 124.2±15.4 to 462.6±81.7 nmol/L) and bicarbonate buffer (HCO3−5% CO2, 1.5 mmol/L Ca2+, approximate increase in [Ca2+] from 79.7±8.7 to 432.2±82.9 nmol/L). By visualizing the experiment on a television monitor and subsequently analyzing videotape and relating these to the emitted indo 1 fluorescence record, the specific PMN-endothelial interaction leading to the rapid rise in endothelial [Ca2+], could be determined. In assays performed in capillary tubes, the PMN-endothelial interaction leading to an increase in endothelial [Ca2+] was linked to firm leukocyte adhesion, as demonstrated by the subsequent resistance of the PMN to detachment despite arterial levels of shear stress (13.4 dyne cm2). This phenomenon was observed regardless of whether PMNs were prestimulated with leukotriene B4 (200 ng/mL×15 min, n=3, not shown). When PMN contact did not elicit an increase in endothelial [Ca2+], PMNs did not resist detachment during subsequent exposure to shear stress in experiments performed in capillary flow tubes.

U937 Monocyte Adhesion Increases Human and Bovine Aortic Endothelial [Ca2+]

To determine whether the increase in endothelial [Ca2+], during contact with PMNs was specific for the PMN-endothelial interaction, adhesion of the human monocytic cell line U937 to both human and bovine aortic endothelial cells was studied. This cell line has been used in adhesion assays as a model of the blood-borne monocyte.28 Contact of U937 cells to human aortic endothelial cells leading to firm adhesion caused a rapid, reversible fourfold increase in [Ca2+] (approximate increase in [Ca2+] from 95.8±24.1 to 404.1±171.2 nmol/L). Similarly, a threefold to fivefold increase in [Ca2+] was observed on initial contact of U937 monocytes with monolayers of bovine aortic endothelial cells (Fig 3). This was observed
regardless of whether endothelial cells were prestimulated with interleukin-1 (15 U/mL × 2 to 4 h, n=4, not shown). Following subsequent exposure to U937 monocytes, the same endothelial monolayer demonstrated repetitive spikes in [Ca\(^{2+}\)] (Fig 3).

**Interaction With Inert Microspheres Does Not Alter Human Aortic Endothelial [Ca\(^{2+}\)]**

To determine whether the increase in endothelial [Ca\(^{2+}\)], observed during PMN or U937 monocyte contact was due to mechanical stimulation of the endothelial membrane, biologically inert 18.25-μm polystyrene microspheres (Coulter Co) were allowed to interact with endothelial monolayers in the same manner described above. While almost all microspheres that contacted the endothelial monolayer were easily detached at even the lowest level of shear stress, resistance to detachment at all levels of shear stress was occasionally observed. These adherent microspheres (n=4, not shown) did not result in a change in endothelial [Ca\(^{2+}\)].
indicating that physical contact alone with a particle of similar size and shape is insufficient to trigger the increase in endothelial \([Ca^{2+}]_i\), and that cell-cell contact is required for this phenomenon to occur.

**Interaction With Endothelial Cells in Suspension and With Red Blood Cells Does Not Alter Human Aortic Endothelial \([Ca^{2+}]_i\)**

To further define the nature of the cell-cell interaction required to elicit an increase in endothelial \([Ca^{2+}]_i\), two cell types that lack the \(\beta 2\) integrin were studied. In experiments similar to those described above, freshly isolated red blood cells (\(n=5\)) or suspensions of cultured human aortic endothelial cells (not loaded with indo 1, \(n=8\)) were perfused through the capillary tube containing the endothelial monolayer and allowed to contact the monolayer under “no-flow” conditions. Before being perfused through the capillary tube containing the endothelial monolayer, endothelial cells were nonenzymatically detached from tissue culture dishes using 1 mmol/L EGTA for 20 minutes and were then resuspended in buffer containing 1.5 mmol/L \(Ca^{2+}\). Approximately 5 to 10 endothelial cells and 20 to 30 red blood cells were used for each endothelial cell in the monolayer.

Increases in endothelial \([Ca^{2+}]_i\) were never observed during these experiments (data not shown), whereas they were noted during subsequent neutrophil attachment. During all experiments with endothelial cell suspensions, some cells subsequently resisted detachment at all levels of shear stress. In contrast, all red blood cells were promptly removed during exposure to even the lowest level of shear stress.

**Effect of Leukocyte Contact on Human Aortic Endothelial \([Ca^{2+}]_i\), in \(Ca^{2+}\)-Free Buffer**

To determine whether the rapid increase in endothelial \([Ca^{2+}]_i\) during leukocyte adhesion was due to \(Ca^{2+}\) influx from the extracellular space or to release from an intracellular \(Ca^{2+}\) store, the effect of leukocyte adhesion on human aortic endothelial \([Ca^{2+}]_i\) was examined in \(Ca^{2+}\)-free buffer, in which influx of \(Ca^{2+}\) from the extracellular space does not contribute to an increase in \([Ca^{2+}]_i\). In these experiments, monolayers were superfused for several minutes in buffer without added \(Ca^{2+}\) and with 1 mmol/L EGTA, resulting in a fall in the indo 1 fluorescence ratio. After stabilization of the indo 1 ratio in \(Ca^{2+}\)-free buffer, leukocytes in \(Ca^{2+}\)-free buffer were allowed to enter the capillary tube or Lucite chamber as previously described. Removal of extracellular \(Ca^{2+}\) did not affect the rapid increase in endothelial \([Ca^{2+}]_i\), during interaction with PMNs (Fig 4; approximate increase in \([Ca^{2+}]_i\), from 86.1±10.0 to 410.7±86.6 nmol/L) or with U937 monocytes (approximate increase in \([Ca^{2+}]_i\), from 76.1±9.0 to 265.0±48.6 nmol/L, not shown). The relatively brief period of buffer \(Ca^{2+}\) removal did not affect the morphological integrity of the endothelial monolayer.

**Depletion of an Intracellular \(Ca^{2+}\) Store Inhibits the Increase in Endothelial \([Ca^{2+}]_i\), During Leukocyte Contact**

To further characterize the contribution of an intracellular \(Ca^{2+}\) store to the increase in endothelial \([Ca^{2+}]_i\), studies were performed using thapsigargin (Tg), which has recently been shown to deplete an endoplasmic reticulum (ER) \(Ca^{2+}\) store in vascular endothelial cells by irreversibly inhibiting ER \(Ca^{2+}\)-ATPase activity.29,30
When human aortic endothelial monolayers were exposed to Tg in the presence of buffer Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\) gradually increased and was maintained at a new level higher than baseline. Exposure to Tg abolished the increase in endothelial [Ca\(^{2+}\)] during interaction with PMNs (Fig 5) or with U937 monocytes. Since depletion of the major portion of the intracellular Ca\(^{2+}\) pool appears to lead to extracellular Ca\(^{2+}\) influx in some cell types, including vascular endothelial cells,\(^2\) experiments were performed in which depletion of the ER Ca\(^{2+}\) store by Tg occurred in the absence of buffer Ca\(^{2+}\). Under these experimental conditions, the sustained phase of the increase in [Ca\(^{2+}\)] initiated by Tg in human aortic endothelial cells is inhibited, as it is in bovine aortic endothelial cells.\(^2\) Fig 6 shows that prior depletion of Tg-sensitive intracellular Ca\(^{2+}\) stores in the absence of buffer Ca\(^{2+}\) also abolished the increase in endothelial [Ca\(^{2+}\)] during interaction with PMNs. Thus,
leukocyte adherence appears to mobilize Ca$^{2+}$ from a Tg-sensitive intracellular pool in endothelial cells, indicating that the ER is probably the source for the increase in [Ca$^{2+}$]i observed on leukocyte interaction with endothelial monolayers.

**Pretreatment of PMNs With an MAb to CD-18 Inhibits the Increase in Human Aortic Endothelial [Ca$^{2+}$]i During Leukocyte Contact**

To examine whether an interaction involving adhesion proteins located on PMNs plays a role in the increase in endothelial [Ca$^{2+}$], PMNs were pretreated with R15.7, a potent and specific MAb to the PMN adhesion protein CD-18. When PMNs were pretreated with R15.7 at a concentration expected to saturate PMN CD-18 (40 µg/mL), a greater number of 10-minute static adhesion periods were necessary to observe a PMN that resisted detachment during subsequent exposure to shear stress. This is not surprising, since a concentration of 20 µg/mL has been shown to block PMN adhesion to human umbilical vein endothelial monolayers by >90%. When adhesion was observed, the increase in endothelial [Ca$^{2+}$]i on PMN contact was inhibited by PMN pretreatment with R15.7. While under control conditions endothelial [Ca$^{2+}$]i increased approximately fourfold (124.2 ± 15.4 to 462.6 ± 81.7 nmol/L >340 nmol/L over baseline) on initial PMN contact, only an approximately twofold increase was observed on contact with R15.7-treated PMNs (115.3 ± 10.8 to 267.8 ± 35.5 nmol/L >150 nmol/L over baseline, Fig 7). These results suggest that the endothelial [Ca$^{2+}$]i increase is in part dependent on a CD-18-mediated adhesion event.

**Discussion**

This report shows that a rapid increase in human and bovine aortic endothelial [Ca$^{2+}$], occurs during the initial contact of human peripheral blood PMNs or U937 monocytes that leads to subsequent firm adhesion. This increase in [Ca$^{2+}$]i occurs within seconds of leukocyte contact and results from release of Ca$^{2+}$ from a Tg-sensitive intracellular store, most likely the endothelial ER. By use of glass capillaries to document resistance of leukocytes to detachment by arterial levels of hemodynamic shear stress, this study establishes a link between firm attachment of leukocytes and mobilization of an intracellular Ca$^{2+}$ store in arterial endothelium.

The rapid release of an intracellular Ca$^{2+}$ store in human and bovine aortic endothelial cells during interaction with human PMNs or human monocytes is not due simply to mechanical stimulation of the endothelial cell, as it did not occur during interaction with inert microspheres. Furthermore, these data suggest that the increase in endothelial [Ca$^{2+}$], on leukocyte-endothelial contact may be initiated by specific adhesion proteins, as no increase in endothelial [Ca$^{2+}$], occurred in experiments with cell lines that lack the β2 integrin and the endothelial [Ca$^{2+}$], increase was inhibited when PMNs were pretreated with a specific MAb to CD-18. The rapid mobilization of [Ca$^{2+}$], may play a critical role in cell adhesion to the endothelium by reinforcing the weak interaction between leukocytes and initially non-stimulated endothelial cells. A similar increase in endothelial [Ca$^{2+}$], on the initial interaction with melanoma cells appears to enhance melanoma cell adhesion to the endothelium. Moreover, several studies have shown that Ca$^{2+}$ channel antagonists inhibit leukocyte adhesion and chemotaxis.

Huang et al also documented an increase in endothelial [Ca$^{2+}$], during PMN adhesion, but this appeared to regulate transendothelial migration of PMNs by increasing monolayer permeability rather than having an effect on PMN adhesion itself. Of note, when Huang et al added PMNs to endothelial monolayers in the absence of chemotactant, no change in endothelial [Ca$^{2+}$], was observed. This contrasts with the results reported in the present study in which endothelial
[Ca\(^{2+}\)], increased during leukocyte contact regardless of whether endothelial cells were prestimulated or PMNs were activated with chemotacticant. The finding that leukocytes bind to nonstimulated endothelial cells in the present study is not surprising, as this has been shown to occur with both PMNs\(^{10,11,33}\) and monocytes.\(^{12,21,34}\) There are differences between the present study and that of Huang et al.\(^7\) which may account for these discrepancies. Huang et al.\(^7\) examined human umbilical vein endothelial cell (HUVEC) monolayers, which may have properties different from the arterial endothelium examined in the present report. In addition, these HUVEC monolayers were grown on human amnion tissue rather than on gelatin-coated glass, as in the present study.

The increase in endothelial [Ca\(^{2+}\)], may enhance cell-cell adhesion through its effect on the expression of adhesion molecules on the endothelium. Rapid, Ca\(^{2+}\)-dependent translocation of P-selectin to the membrane\(^{16,17}\) may result initially in loose adhesion of leukocytes, which then become tightly adherent through an interaction between CD11/CD18 integrins on leukocytes and their counterreceptor, ICAM-1. Recently, Ca\(^{2+}\) ionophore has been shown to act synergistically with an activator of PKC to upregulate ICAM-1 expression on endothelial cells.\(^18\) Another recent report suggests that leukocyte adhesion itself induces synthesis of ICAM-1 by endothelial cells.\(^35\) Adhesion of monocytes induced endothelial ICAM-1 mRNA by 4 hours and increased ICAM-1 expression on endothelial cells by 14 hours after adhesion.\(^35\)

The increase in endothelial [Ca\(^{2+}\)], on contact with leukocytes is mirrored by an increase in leukocyte [Ca\(^{2+}\)], during this interaction. Adherent PMNs demonstrate transient increases in [Ca\(^{2+}\)]\(^{11,12}\); transmembrane signaling by the T3-antigen receptor complex mobilizes an InsP\(_3\)-sensitive intracellular Ca\(^{2+}\) store in lymphocytes;\(^4\) an increase in monocyte [Ca\(^{2+}\)], regulates the high-affinity receptor function of Ca\(^{2+}\)\(^{11}\); and cross-linking of the differentiation protein CD14 on monocytes increases monocyte [Ca\(^{2+}\)], and enhances adhesion to stimulated endothelial cells.\(^6\) A recent report also demonstrates that sulfatides, ligands for leukocyte L-selectin, trigger an increase in PMN [Ca\(^{2+}\)], through ligation of L-selectin.\(^36\) This effect was strictly dependent on sulfation of galactose and was inhibited by prior shedding of PMN L-selectin.\(^36\) Thus, an increase in [Ca\(^{2+}\)], in both the leukocyte and the endothelial cell appears to be an important early signaling event in leukocyte-endothelial adhesion.

The increase in [Ca\(^{2+}\)], during the leukocyte-endothelial interaction may have clinical importance, as Schroeder et al.\(^27\) recently reported that the Ca\(^{2+}\) channel blocker diltiazem inhibited the development of coronary artery disease in heart transplant recipients, a process that may be initiated by interaction of circulating lymphocytes and the vascular endothelium of the transplanted heart. Ca\(^{2+}\) channel blockers have also been shown to inhibit LDL-stimulated formation of InsP\(_3\) in human lymphocytes, which may in turn have an effect on ER Ca\(^{2+}\) release. The mechanism of the inhibition of PMN adhesion and chemotaxis by Ca\(^{2+}\) channel antagonists\(^{31,32}\) is incompletely understood, but these studies in combination suggest that Ca\(^{2+}\) antagonists may well have effects on intracellular Ca\(^{2+}\) homeostasis in leukocytes in addition to their well-known effects on Ca\(^{2+}\) channels in other cell types. Thus, pharmacological manipulation of leukocyte or endothelial [Ca\(^{2+}\)], in situations in which leukocyte adhesion and subsequent leukocyte-mediated injury are likely to occur, such as following coronary angioplasty or reperfusion of ischemic myocardium, may provide a novel therapeutic strategy in the treatment of cardiovascular disease.

References


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