Calcium mobilization and Rac1 activation are required for VCAM-1 (vascular cell adhesion molecule-1) stimulation of NADPH oxidase activity

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VCAM-1 (vascular cell adhesion molecule-1) plays an important role in the regulation of inflammation in atherosclerosis, asthma, inflammatory bowel disease and transplantation. VCAM-1 activates endothelial cell NADPH oxidase, and this oxidase activity is required for VCAM-1-dependent lymphocyte migration. We reported previously that a mouse microvascular endothelial cell line promotes lymphocyte migration that is dependent on VCAM-1, but not on other known adhesion molecules. Here we have investigated the signalling mechanisms underlying VCAM-1 function. Lymphocyte binding to VCAM-1 on the endothelial cell surface activated an endothelial cell calcium flux that could be inhibited with anti- α 4-integrin and mimicked by anti-VCAM-1-coated beads. VCAM-1 stimulation of calcium responses could be blocked by an inhibitor of intracellular calcium mobilization, a calcium channel inhibitor or a calcium chelator, resulting in the inhibition of NADPH oxidase activity. Addition of ionomycin

overcame the calcium channel blocker suppression of VCAM-1-stimulated NADPH oxidase activity, but could not reverse the inhibitory effect imposed by intracellular calcium blockage, indicating that both intracellular and extracellular calcium mobilization are required for VCAM-1-mediated activation of NADPH oxidase. Furthermore, VCAM-1 specifically activated the Rho-family GTPase Rac1, and VCAM-1 activation of NADPH oxidase was blocked by a dominant negative Rac1. Thus VCAM-1 stimulates the mobilization of intracellular and extracellular calcium and Rac1 activity that are required for the activation of NADPH oxidase.

Key words: calcium channel, Cdc42, intracellular calcium release, NADPH oxidase, Rac1, vascular cell adhesion molecule-1 (VCAM-1).

INTRODUCTION

VCAM-1 (vascular cell adhesion molecule-1) expression is induced on endothelial cells in inflammatory tissues during atherosclerosis, immune challenge and transplantation [1–4]. Prior to atherosclerotic lesion formation, VCAM-1 is expressed on endothelial cells in sites predisposed to lesion formation [1]. VCAM-1 is also induced on smooth muscle cells and lymphoid follicular dendritic cells. Induction of VCAM-1 expression on smooth muscle cells has been hypothesized to play a role in their differentiation [5]. VCAM-1 on follicular dendritic cells may regulate B cell function [6]. In contrast, VCAM-1 is constitutively expressed by lymph node HEV (high endothelial venule) cells *in vivo* and by HEV-derived cultured endothelial cells [7,8]. VCAM-1 also has an important function during fetal development, since disruption of the first domain of the VCAM-1 gene results in loss of the embryo [9]. Adhesion to VCAM-1 is required for T cell or eosinophil migration into certain inflammatory sites [3,4,10,11].

Little is known regarding the molecular mechanisms responsible for VCAM-1 signal transduction. As we have shown previously, lymphocyte adhesion to VCAM-1 and the subsequent signals activated by VCAM-1 are required for lymphocyte migration [12]. The migration process at a distinct site occurs within minutes, suggesting that VCAM-1 is capable of mediating rapid cellular responses. Ligand binding to VCAM-1 stimulates the production by NADPH oxidase of a low concentration (1 μ M) of ROS (reactive oxygen species) that is 30–200-fold less than that produced by activated macrophages and neutrophils [13]. This NADPH oxidase activity is required for VCAM-1-depend-

ent lymphocyte migration, but not for lymphocyte adhesion [12].

To investigate the mechanism behind the VCAM-1-mediated activation of NADPH oxidase, we have focused on two possible signalling components downstream of VCAM-1: intracellular Ca^{2+} and the Rac1 GTPase. The former is known to be activated by antibody cross-linking of VCAM-1 on both TNF α (tumour necrosis factor α)-stimulated HUVECs (human umbilical vein endothelial cells) and an endothelial cell line derived from HUVECs [14,15], whereas the latter is a critical part of the neutrophil NADPH oxidase complex [16–18]. We found that VCAM-1-dependent lymphocyte stimulation of calcium responses could be blocked by an intracellular calcium mobilization inhibitor or a calcium channel inhibitor, as well as by a calcium chelator, resulting in the inhibition of VCAM-1-mediated activation of NADPH oxidase activity. Furthermore, VCAM-1 specifically activated the Rho-family GTPase Rac1 that is required for the VCAM-1-mediated signals. Our results suggest that stimulation of VCAM-1 leads to the mobilization of intracellular and extracellular calcium and to elevated Rac1 activity, the combined signals of which may be required for the activation of NADPH oxidase during lymphocyte migration.

MATERIALS AND METHODS

Cells

BALB/c mice were used in accordance with institutional guidelines. For each assay, spleen cells from at least three BALB/c mice

Abbreviations used: AM, acetoxymethyl ester; BAPTA AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); EGFP, enhanced green fluorescent protein; DHR 123, dihydrorhodamine 123; GST, glutathione S-transferase; HEV, high endothelial venule; HUVEC, human umbilical vein endothelial cell; PAK, p21-activated kinase; ROS, reactive oxygen species; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; TNF α , tumour necrosis factor α ; VCAM-1, vascular cell adhesion molecule-1; WASP, Wiskott–Aldrich syndrome protein.

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(4–6 weeks old; Harlan Industries, Indianapolis, IN, U.S.A.) were isolated as described previously [7]. Spleen red blood cells were lysed as described previously [12]. The endothelial cell line mHEVa, generated in this laboratory, was cultured in mouse endothelial cell medium with 20 % (v/v) heat-inactivated fetal calf serum [7]. Cell viability after each treatment was determined by Trypan Blue exclusion.

Antibody-coated beads

Streptavidin-conjugated 10.4 μm beads (Bangs Laboratories, Inc., Fishers, IN, U.S.A.) were coated with biotin-conjugated goat anti-(rat Ig), washed, and then incubated with rat anti-(mouse VCAM-1) as described previously [12]. These beads were not phagocytosed even though these cells are capable of phagocytosis of apoptotic cells [19].

Transfection of endothelial cells

mHEVa cells were grown to 90 % confluence in six-well plates and transfected with 5 μg of the EGFP (enhanced green fluorescent protein) vector pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) alone, vector with dominant negative N17-Rac1 with N-terminal EGFP, or vector with dominant negative N17-Cdc42 with N-terminal EGFP. Each of these transfections was performed in the presence of 7 or 15 μl /well lipofectAMINE 2000TM Reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions, in culture medium without gentamicin, penicillin or streptomycin. After 6 h, the medium was removed and replaced with medium containing antibiotics. At 24 h, the cells were suspended by treatment with 0.03 % EDTA in PBS, washed and plated in 100 mm Petri dishes. After 48 h, the medium was removed and cells were treated with 0.3 μg of geneticin/ml of medium (Invitrogen), a concentration that is completely toxic to non-transfected mHEV cells within 72 h. After growth to 75 % confluence, the cells were suspended in 0.03 % EDTA and washed, and fluorescence was examined by flow cytometry. Approx. 20 % of these cells were EGFP-positive, as determined by flow cytometry. Therefore the cells were subcloned twice at a cell density of one-third of a cell/well (i.e. one cell every three wells) to generate clones with homogeneous expression of EGFP, and then subcloned once more to determine whether all subclones were EGFP-positive as determined by flow cytometry. Clones with EGFP vector were designated mHEVa63 and mHEVa4425. Clones with EGFP-N17-Rac1 were designated mHEVa801rac and mHEVa7595rac. Clones with EGFP-N17-Cdc42 were designated mHEVa7711cdc, mHEVa7715cdc and mHEVa7611cdc.

For morpholino-antisense transfections, endothelial cells grown in 24-well plates to 70 % confluence were transfected with carboxyfluorescein-labelled morpholino-antisense oligomers to gp91 phox (5'-CTTCATTCACAGCCAGTTCCCCAT-3') or a carboxyfluorescein-labelled standard control morpholino-oligomer (5'-CCTCTTACCTCAgTTACAATTTATA-3') according to the protocol from GENE TOOLS, LLC (Philomath, OR, U.S.A.). For each well, FITC-labelled morpholino-oligomers (0.465 nmol) were incubated for 20 min with 31.3 μl of water and 0.93 μl of ethoxylated polythylenimine. After 20 min, the morpholino-oligomer solution was mixed with 500 μl of serum-free medium. Then 500 μl of this solution was added per well of endothelial cells for 1 h. After transfection, the medium was removed from the cells and replaced with fresh medium containing 20 % (v/v) fetal calf serum. After 3 days, gp91 phox protein expression was examined by Western blot using a mouse anti-(mouse gp91)

antibody (a gift from Dr D. Roos and Dr E. van der Schoot, University of Amsterdam, The Netherlands) (1:200) followed by a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Amersham) and an ECL plus[®] kit (Amersham).

ROS

Generation of ROS was examined as reported previously by us [20]. Briefly, endothelial cells were incubated in 1 μM DHR 123 (dihydrorhodamine 123) (Molecular Probes) in Phenol Red-free medium for 15 min, and then anti-VCAM-1-coated beads, lymphocytes or H_2O_2 standards were added. DHR 123 is oxidized by H_2O_2 (but not superoxide) in the presence of cellular peroxidases and becomes fluorescent [21]. Accumulation of fluorescence was examined for 0–60 min by time lapse confocal microscopy and quantified by calculating the sum of the pixel intensities/100 μm^2 and then regression analysis using a standard curve. VCAM-1 stimulates detectable accumulation of rhodamine fluorescence by 10 min, with a maximum accumulation at 30 min. Maximum accumulation of ROS generation at 30 min is presented.

Calcium flux

Endothelial cells were incubated with the calcium indicator indo-1 AM (acetoxymethyl ester) (5 μM) in 0.05 % Pluronic F-127 in endothelial cell medium containing 5 % (v/v) fetal calf serum for 30 min at room temperature, followed by 30 min at 37 °C, to achieve adequate loading of the endothelial cells. The AM form of indo-1 is membrane-permeant; it is hydrolysed in the cytoplasm to the impermeant trapped form. The endothelial cells were washed and suspended in Phenol Red-free endothelial cell medium containing 20 % (v/v) fetal calf serum [12]. These endothelial cells were activated as indicated while examining them every 2 s by confocal microscopy with UV laser excitation. The ratio of the sum of the emission fluorescent intensities at 405 nm and 490 nm was calculated. Calcium concentrations were not determined, since the indo-1 fluorescence intensity for calcium concentrations inside cells is different from that for calcium in standard curve solutions [22]. Previous reports indicate that antibody cross-linking of VCAM-1 stimulates a 190 nM increase in endothelial cell calcium [14]. A focus of the present paper is the function of the calcium response upon VCAM-1 stimulation.

Propidium iodide labelling of hypodiploid nuclei [23]

Endothelial cells (2×10^6) were washed in PBS (4 °C), fixed in 2 ml of 70 % (v/v) ethanol (4 °C) for 30 min, washed in 10 ml of PBS (4 °C), suspended in DNA staining reagent [PBS, 0.1 mM EDTA and 0.05 mg/ml RNase A (50 units/mg; protease- and DNase-free; Boehringer-Mannheim, Indianapolis, IN, U.S.A.), pH 7.4] containing 50 μg /ml propidium iodide (Sigma) at room temperature for 15 min, and then analysed by flow cytometry.

Endogenous Rac1 and Cdc42 activity assay

For each sample, 25 μg of anti-VCAM-1 (clone MVCAM.A; PharMingen, San Diego, CA, U.S.A.) and 10 μg of goat anti-(rat Ig) (Southern Biotechnology, Birmingham, AL, U.S.A.) in 135 μl of endothelial cell culture medium were incubated for 10 min at room temperature. These antibodies were added to a confluent monolayer of endothelial cells in a 60 mm Petri dish to stimulate VCAM-1. At the times indicated, the medium was removed, the cells were placed on ice, 1 ml of suspension buffer (20 mM Tris/HCl, 5 mM MgCl_2 , 20 mM NaCl, 1 mM dithiothreitol and Sigma

protease inhibitor cocktail #P2714) was added and the samples were frozen overnight at -80°C . Active Rac1 and Cdc42 in the same set of samples were determined as described previously [24]. Briefly, freshly thawed cells on plates were lysed by incubation on ice for 30 min in the suspension buffer supplemented with 1 % Triton X-100. A 5 μg portion of GST (glutathione S-transferase), GST-PAK1 (p21-activated kinase) or GST-WASP (Wiskott-Aldrich syndrome protein) immobilized on glutathione-agarose beads were added to the lysates. After 45 min of agitation at 4°C , the beads were washed twice with 25 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl_2 , and 40 mM NaCl plus 0.5 % Nonidet P40, and then three times with this buffer without detergent. Total Rac1 and Cdc42 in the cell lysates as well as the GTP-bound forms of Rac1 and Cdc42 co-precipitated with GST-PAK1 or GST-WASP were revealed by anti-Rac1 or anti-Cdc42 Western blotting.

Statistical analysis

Data were analysed by ANOVA followed by a Tukey's multiple comparisons test (Sigma Stat; Jandel Scientific).

RESULTS

Lymphocyte adhesion to VCAM-1 activates a calcium response

We examined whether lymphocyte binding to VCAM-1 activates a calcium response, whether VCAM-1 activates calcium channels and/or an intracellular calcium release, and whether calcium functions are important for VCAM-1 signals through NADPH oxidase. To address these questions, murine microvascular endothelial cell lines (mHEVa) were used that constitutively express VCAM-1 [7]. This constitutive VCAM-1 expression avoided complications in signals used to induce VCAM-1 expression. In addition, cell lines were important for the generation of the stable transfectants discussed in this paper. Since the mHEV cell lines utilize only VCAM-1 and not other known adhesion molecules to bind lymphocytes, they were used to determine whether lymphocyte binding to the endothelial cells activates a calcium response [12,13]. The mHEVa cells were preloaded with the calcium indicator indo-1 AM (5 μM), washed and incubated with spleen lymphocytes. Lymphocytes stimulated a calcium response in the mHEVa cells (Figure 1). This response was blocked by inhibition of lymphocyte binding with antibodies directed against $\alpha 4$ -integrin, the VCAM-1 ligand on lymphocytes (Figure 1). As a positive control, the inhibition was overcome by subsequent addition of ionomycin (results not shown).

We also determined whether antibody cross-linking of VCAM-1 with antibody-coated beads activates a calcium response. To cross-link VCAM-1, indo-1-loaded mHEVa cells were stimulated with 10 μm beads that were coated with anti-VCAM-1 antibodies or isotype control antibodies. A calcium response was stimulated in the mHEVa cells by anti-VCAM-1-coated beads, but not by isotype antibody-coated beads (Figure 2). This is consistent with calcium responses stimulated by soluble antibody cross-linking of VCAM-1 on HUVECs [14]. The more prolonged calcium response to antibody cross-linking of VCAM-1 as compared with that induced by lymphocyte binding is likely to reflect the more prolonged antibody binding to VCAM-1 as compared with the asynchronous transient lymphocyte binding to VCAM-1 that occurs as the lymphocytes migrate across the apical surface of endothelial cells. Nevertheless, anti-VCAM-1-coated beads can be used instead of lymphocytes for studies using reversible pharmacological inhibitors that would have otherwise affected the lymphocytes as well.

Lymphocyte activation of VCAM-1 stimulates intracellular calcium release as well as calcium channels

It is not known whether intracellular calcium release and/or calcium channels are involved in VCAM-1 signals. mHEVa cells were preloaded with indo-1 AM and incubated with inhibitors of intracellular calcium release {TMB-8 [8-(diethylamino)octyl-3,4,5-trimethoxybenzoate]} or calcium channels (verapamil) and then stimulated with lymphocytes. Intracellular calcium was also chelated by loading the cells with BAPTA AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)]. As a control, lymphocyte binding was blocked with anti- $\alpha 4$ -integrin antibodies. Anti- $\alpha 4$ -integrin, TMB-8, verapamil and BAPTA AM reduced the magnitude of the calcium response significantly (Figure 1). Shown are the optimal doses for the inhibitors. These doses have been reported previously for inhibition of other calcium-dependent responses in endothelial cells [25–29] (dose-dependent effects of BAPTA on VCAM-1 signals are shown in Figure 4). Others have reported a 10–200 μM BAPTA concentration range for inhibition of calcium responses in endothelial cells, with 50 μM being a typical concentration for inhibition [26,27,30]. The inhibitors did not affect VCAM-1 expression, as determined by immunolabelling and flow cytometry (results not shown). In summary, VCAM-1 stimulates both intracellular release of calcium and calcium channels.

Calcium is necessary for VCAM-1-stimulated 'outside-in' signals that are required for VCAM-1-dependent lymphocyte migration

It was determined whether calcium is involved in an endothelial cell function that is required for VCAM-1-dependent lymphocyte migration, i.e. VCAM-1-mediated activation of endothelial cell NADPH oxidase [12]. NADPH oxidase activity is not required for lymphocyte adhesion to VCAM-1, allowing the investigation of signals required for migration independently of requirements for the lymphocyte adhesion event [12]. mHEVa cells were preloaded for 15 min with DHR 123, which when oxidized by H_2O_2 in the presence of cellular peroxidase becomes fluorescent. We have demonstrated previously that, in response to VCAM-1, stimulation of DHR fluorescence is mediated by NADPH oxidase, but not by other ROS-generating enzymes [12]. In addition, a gp91 phox morpholino-antisense oligomer blocked gp91 phox expression and VCAM-1-stimulated DHR fluorescence (Figure 3), demonstrating the function of NADPH oxidase in this response. To chelate intracellular calcium, endothelial cells were loaded with BAPTA AM for 15 min and washed. VCAM-1 was stimulated by lymphocyte binding or anti-VCAM-1-coated beads. BAPTA chelation of endothelial cell calcium blocked activation of endothelial cell NADPH oxidase (Figure 4).

Next we determined whether intracellular calcium release or calcium channels are involved in activation of NADPH oxidase by VCAM-1. The endothelial cells were treated with TMB-8 or verapamil for 15 min, DHR was added for the next 15 min and then the endothelial cells were stimulated with anti-VCAM-1-coated beads. The endothelial cells were not stimulated with lymphocytes, since the reversible inhibitors left in the culture might also affect lymphocytes. TMB-8 and verapamil did not alter DHR loading of the endothelial cells, as background fluorescence was the same as in non-treated cells (Figure 5). TMB-8 and verapamil blocked VCAM-1 activation of ROS production (Figure 5). It was then determined whether the ionophore ionomycin could overcome the inhibition of VCAM-1-stimulated ROS production by TMB-8 and verapamil. Ionomycin stimulation alone did not activate ROS production and did not affect background ROS produced by endothelial cell metabolism

Figure 1 Lymphocytes stimulate an endothelial cell calcium flux that is dependent on $\alpha 4$ -integrin adhesion, intracellular calcium release and calcium channels

Confluent monolayers of mHEV cells were loaded with the calcium indicator indo-1 AM (5 μ M), washed and then treated with TMB-8 (40 μ M), verapamil (50 μ M) or BAPTA AM (50 μ M) for 30 min. These doses have been used previously for inhibition of other receptor-induced calcium responses in endothelial cells [25–27]. The endothelial cells were stimulated with 4×10^6 lymphocytes (L) that had been preincubated for 5 min in the presence or absence of 4 μ g of anti- $\alpha 4$ -integrin antibodies. Indo-1 fluorescence was examined by confocal microscopy. (A) Representative responses from at least three experiments: (a) lymphocytes; (b) lymphocytes and anti- $\alpha 4$ -integrin antibodies; this level of antibody blocks adhesion, as we reported previously [55] (results not shown); (c) ionomycin (2 μ M), a positive control; (d) lymphocytes and TMB-8 (40 μ M); (e) lymphocytes and verapamil (50 μ M); (f) lymphocytes and BAPTA AM (50 μ M). Shown are representative calcium responses of three experiments with the optimal inhibitor doses. Arrows indicate the time of addition of the stimulants. (B) Data from triplicate experiments at the optimal inhibitor doses are presented as the magnitude of the calcium response (peak height; mean $\pm$ S.E.M.) and the length of the calcium response (maximum peak width). Peak width was from initiation of the peak to when the peak returned to baseline. The inhibitors had no effect on cell viability, as determined by Trypan Blue exclusion (results not shown). * $P < 0.05$ compared with the lymphocyte-stimulated control.

(Figure 5). Ionomycin did reverse the verapamil-mediated inhibition of VCAM-1 stimulation, but did not reverse the inhibition by TMB-8 (Figure 5). In summary, both intracellular calcium release and calcium channels are necessary for activation of endothelial cell NADPH oxidase by VCAM-1.

VCAM-1 activates Rac1, but not Cdc42, and this mediates stimulation of endothelial cell NADPH oxidase

Leucocyte NADPH oxidase activation involves the Rho-family small GTPase Rac1. Therefore we hypothesized that Rac1 may contribute to VCAM-1 signals for the activation of endothelial

cell NADPH oxidase. The possible involvement of another Rho protein, Cdc42, was examined in parallel, because it has been reported to have opposing effects to Rac1 in endothelial cells in response to thrombin-induced permeability changes [31]. Lymphocyte induction of Rac activation in the endothelial cells was not tested because, after adhesion, the assay would detect the combination of Rac activity in lymphocytes and in endothelial cells. Therefore, to examine VCAM-1 stimulation, VCAM-1 on the surface of endothelial cells was cross-linked with anti-VCAM-1 followed by a secondary antibody, and the activation status of endogenous Rac1 and Cdc42 was determined by an effector domain (PAK1 or WASP) pull-down assay. At 30–60 s after cross-linking, anti-VCAM-1 stimulated a significant increase in

Figure 2 A calcium flux is stimulated by anti-VCAM-1-coated beads, but not by isotype-coated beads

Confluent monolayers of mHEvA cells were loaded with 5 μ M indo-1 AM, stimulated with antibody-coated beads (10.4 μ m) and examined by confocal microscopy. (A) Anti-VCAM-1-coated beads; (B) isotype antibody-coated beads. Representative responses of at least two experiments are shown. Arrows indicate the time for addition of the antibody-coated beads.

Figure 3 VCAM-1 stimulates gp91 phox-containing NADPH oxidase

Monolayers of mHEvA cells (60% confluent) were ethoxylated-polythylenimine-transfected with gp91 phox morpholino-antisense or a control morpholino-oligomer and incubated for 72 h. These cells were examined for VCAM-1 activation of rhodamine fluorescence (A). Data are presented as means $\pm$ S.D. from two experiments; * P < 0.05 compared with control morpholino-oligomer-treated cells. (B) Lower panel: gp91 phox expression was examined by Western blot. Equal protein was loaded in the lanes. Lane 1, control morpholino-treated cells. Lane 2, gp91 phox morpholino-antisense-treated cells. The arrow indicates gp91 phox. Upper panel: only two non-specific bands were present (200–230 kDa), and these bands demonstrated equal intensity in all lanes. These bands also occurred in control blots with murine macrophages (results not shown). The upper and lower panels shown are from the same blot and are representative of two experiments.

Rac1-GTP species as compared with cells incubated with isotype control antibodies (Figure 6). The Rac1 activity declined after 1 min, which is consistent with the rapid transient function of Rac activity in signal transduction [32,33]. Thus this Rac1 activity occurred before the detection of NADPH oxidase activity. Detectable accumulation of NADPH oxidase-generated ROS in a monolayer of endothelial cells occurred within 10 min, with the maximum accumulation of fluorescence at 30 min, as reported previously by us and others [12,34]. In contrast with Rac1, no change in Cdc42 activity in response to VCAM-1 was detected under similar conditions (Figure 6). These results indicate that VCAM-1 specifically activates Rac1 in this system.

We next determined whether Rac1 or Cdc42 mediates the activation of NADPH oxidase by VCAM-1. We used mHEvA cells

Figure 4 Calcium is required for activation of NADPH oxidase by VCAM-1

Confluent monolayers of mHEvA cells were treated with BAPTA AM (10 or 50 μ M) for 30 min and washed. The endothelial cell monolayers were incubated with DHR 123 for 15 min and then stimulated with lymphocytes or anti-VCAM-1-coated beads. Shown are data from two experiments (means $\pm$ S.D.); a lack of an error bar indicates an S.D. that is too small to distinguish clearly. * P < 0.05 compared with non-treated endothelial cells; # P < 0.05 compared with lymphocyte-stimulated or anti-VCAM-1-stimulated endothelial cells.

Figure 5 Intracellular calcium release and calcium channels are required for activation of endothelial cell NADPH oxidase

Endothelial cells were treated for 15 min with TMB-8 (40 μ M) or verapamil (50 μ M), and then loaded for 15 min with DHR 123. TMB-8 did not affect the loading of DHR 123, since the background fluorescence was not altered after loading (results not shown). The endothelial cells were non-stimulated or were stimulated with anti-VCAM-1-coated beads and/or ionomycin (2 μ M) for 30 min and examined by confocal microscopy. Shown are data from two experiments (means $\pm$ S.D.); + P < 0.05 compared with non-treated non-stimulated endothelial cells. # P < 0.05 compared with non-treated endothelial cells stimulated with anti-VCAM-1-coated beads; * P < 0.05 compared with verapamil-treated cells stimulated with anti-VCAM-1-coated beads. There are no significant differences among the TMB-8-treated groups. There are also no significant differences for TMB-8- or verapamil-treated non-stimulated endothelial cells compared with non-treated non-stimulated endothelial cells.

to generate clones stably expressing dominant negative N17-Rac1 and N17-Cdc42 mutants with an N-terminal EGFP tag. The clones containing EGFP (10–20% efficiency) were selected after three rounds of limiting dilution (one-third of a cell/well) and examined for fluorescence by flow cytometry. Several clones were selected that were >95% EGFP⁺ as determined by flow cytometry, and these clones exhibited a single fluorescent peak (Table 1). The clones displayed similar VCAM-1 expression levels to the wild-type parental cells (results not shown) and had low levels of apoptosis ($8.9 \pm 2.5\%$ of transfected cells were apoptotic, compared with $8.0 \pm 2.3\%$ non-transfected cells), as

Figure 6 Anti-VCAM-1 activates Rac1, but not Cdc42

Confluent monolayers of endothelial cells were stimulated for 0.5, 1, 2 or 12 min with anti-VCAM-1 (V) or an isotype control antibody (I) plus a secondary antibody. Active Rac1 (Rac1-GTP) or Cdc42 (Cdc42-GTP) in cell lysates was isolated using effector domain pull-down assays. These isolates or total cell lysates were subjected to SDS/PAGE. Western blots were performed to detect Rac1 and Cdc42. (A) Data from a representative experiment of three. VCAM-1 stimulated an increase in active Rac1, but there were no changes in active Cdc42, total cell Rac1 or total cell Cdc42. (B) Means $\pm$ S.D. for data from three experiments; * $P < 0.05$ compared with isotype controls.

Table 1 Mean fluorescence intensity of the transfected mHEVa cell lines

The mean fluorescence intensity (MFI) of EGFP was determined by flow cytometry analysis of the cells. WT, wild type.

Transfection	Clone name	MFI
None	WT mHEVa	28
EGFP-N17-Rac1	mHEVa801Rac	1062
	mHEVa7595Rac	254
EGFP-N17-Cdc42	mHEVa7711Cdc	1300
	mHEVa7611Cdc	462
	mHEVa7715Cdc	1210
EGFP vector	mHEVa63v	936
	mHEVa4425v	375

determined by propidium iodide labelling of hypodiploid nuclei and flow cytometry analysis. The cells were $>95\%$ viable, as determined by Trypan Blue exclusion, and had no obvious defects in cell growth or morphology, as determined by light microscopy (results not shown).

These cells expressing Rac1 or Cdc42 mutants were examined for the VCAM-1-stimulated production of ROS. The cells were incubated with DHR for 15 min and stimulated with a confluent monolayer of anti-VCAM-1-coated $10\ \mu\text{m}$ beads as described previously [12]. The mHEVa cells expressing EGFP-N17-Rac1 failed to generate ROS in response to the anti-VCAM-1-coated beads (Figure 7) under conditions where anti-VCAM-1-coated beads readily stimulated ROS production by non-transfected mHEV cells, or mHEV cells transfected with the EGFP control vector or with the EGFP-N17-Cdc42 vector (Figure 7). Therefore VCAM-1 signals depend on Rac1, but

Figure 7 VCAM-1-stimulated Rac1 is required for activation of NADPH oxidase

The transfected clones listed in Table 1 were loaded for 15 min with DHR 123. Loading of DHR 123 was similar for all clones, since the background fluorescence was not altered. The endothelial cells were non-stimulated or were stimulated with anti-VCAM-1-coated beads for 30 min and examined by confocal microscopy. Shown are means $\pm$ S.D. from two experiments. Clones with dominant negative Rac1 had reduced VCAM-1 stimulation of ROS generation as compared with the other endothelial cell clones (* $P < 0.05$).

not Cdc42, for the stimulation of NADPH oxidase that we have reported to be required for the promotion of VCAM-1-dependent lymphocyte migration [12].

DISCUSSION

In the present studies we show that binding of lymphocyte $\alpha 4$ -integrin to VCAM-1 on endothelial cells stimulates an endothelial cell calcium response and Rac1 activation. This response is generated by both the release of intracellular calcium and the functioning of calcium channels. Furthermore, intracellular calcium release, calcium channels and Rac1 are necessary for the VCAM-1-mediated activation of endothelial cell NADPH oxidase, an enzyme required for endothelial cell promotion of VCAM-1-dependent lymphocyte migration. Thus these results identify a role for calcium and Rac1 in VCAM-1 'outside-in' signalling. These VCAM-1 signals are likely to occur in smooth muscle cells and dendritic cells that can be induced to express VCAM-1 [5,6].

In neutrophils, the participation of calcium differs for the activation of plasma membrane compared with granule NADPH oxidase [35]. As discussed by Granfeldt et al. [35], in neutrophils the calcium ionophore ionomycin does not stimulate plasma membrane-associated NADPH oxidase, but does stimulate intracellular granule-associated NADPH oxidase [36]. Consistent with the lack of ionomycin stimulation of plasma membrane NADPH oxidase, we showed that ionomycin alone did not stimulate NADPH oxidase activity in endothelial cells. Furthermore, scavenging ROS, that are produced extracellularly, block detection of VCAM-1-stimulated endothelial cell NADPH oxidase activity and inhibit VCAM-1-dependent lymphocyte migration across a monolayer of endothelial cells [12]. These data suggest that an endothelial cell plasma membrane NADPH oxidase functions during VCAM-1-dependent lymphocyte migration.

The activation of NADPH oxidase has been examined most completely in neutrophils. There are multiple mechanisms for the activation of neutrophil NADPH oxidase, depending on the stimulant [37]. Neutrophil NADPH oxidase can be activated by calcium- and calmodulin-dependent and -independent mechanisms and by protein kinase C-dependent and -independent mechanisms [38,39]. For receptors that utilize calcium during activation of neutrophil NADPH oxidase, extracellular calcium influx and intracellular calcium release have been examined. Calcium regulates chemoattractant fMetLeuPhe-stimulated neutrophil

plasma membrane NADPH oxidase [35]. Removal of extracellular calcium blocks this chemoattractant-mediated stimulation [40]. In contrast, in Fc α R-stimulated neutrophils, intracellular calcium release is sufficient for activation of NADPH oxidase and is independent of store-operated calcium influx [41]. In cell-free systems, NADPH oxidase activity in the presence of phosphatidic acid and diacylglycerol does not require calcium [42]. Therefore calcium may not be necessary for the activity of the NADPH oxidase enzyme complex, but may function in signals upstream of NADPH oxidase for its activation. The signalling intermediates for the activation of endothelial cell NADPH oxidase by VCAM-1 have not yet been identified. Nevertheless, the present study indicates that a calcium-dependent pathway is involved in the activation of endothelial cell NADPH oxidase by VCAM-1, and that this pathway utilizes both calcium channels and intracellular calcium release.

VCAM-1-mediated activation of endothelial cell NADPH oxidase is required for VCAM-1-stimulated endothelial cell cytoskeletal changes [12,34,43]. Calcium has also been reported as being important for endothelial cell cytoskeletal rearrangements. Huang et al. [44] reported that activated neutrophils stimulate both an increase in endothelial cell permeability and a calcium response in endothelial cells that is necessary for neutrophil migration in 15 min across HUVEC monolayers. However, they did not identify the receptors stimulating this calcium response. They hypothesized that calcium may be important for calcium/calmodulin-dependent changes in phosphorylation of myosin light chains by myosin light chain kinase, thus causing an endothelial cell shape change. However, we have reported previously that endothelial cell calmodulin is not required for VCAM-1-dependent lymphocyte migration [12]. Lorenzon et al. [15] reported that endothelial-cell actin stress-fibre formation and endothelial cell calcium are activated by binding of neutrophils to HUVECs via the adhesion molecules VCAM-1, P-selectin and E-selectin, but not ICAM-1 (intercellular cell adhesion molecule-1) or PECAM-1 (platelet/endothelial cell adhesion molecule-1). Our data show that calcium was required for VCAM-1 stimulation of endothelial cell NADPH oxidase, and we and others reported previously that NADPH oxidase activity is required for actin rearrangements [12,43]. Therefore calcium is required for VCAM-1-stimulated cytoskeletal changes, at least through the activation of NADPH oxidase.

It is certainly possible that calcium also functions at other points in the VCAM-1 signalling pathway. H₂O₂ (1–10 μ M) has been reported to stimulate intracellular calcium release [45]. This is in the range of ROS concentrations (1 μ M) produced by VCAM-1 stimulation [20]. Therefore calcium may also function downstream of NADPH oxidase and participate in endothelial cell cytoskeletal changes for endothelial cell retraction and promotion of VCAM-1-dependent leucocyte migration.

Some endothelial cells have L-type calcium channel function, and several endothelial cell responses (such as endothelin release) are inhibited by 10–150 μ M verapamil, indicating that these doses block L-type calcium channels in endothelial cells [28,29]. Verapamil has been reported to have beneficial effects in transplantation, potentially resulting from the modulation of endothelial cells. For example, one report indicated that 50 μ M verapamil inhibited TNF α - or interleukin-1 β -induced VCAM-1 expression on HUVECs [46]. In contrast, Blaheta et al. [47] reported that 50 μ M verapamil did not affect the induction of VCAM-1 expression on HUVECs by interleukin-1 plus TNF α . In our studies, 50 μ M verapamil did not alter constitutive VCAM-1 expression, and therefore this is not the mechanism behind the inhibition by verapamil of the VCAM-1-stimulated calcium response and NADPH oxidase activation. Taken together, these

data suggest that, in transplants treated with verapamil, verapamil can reduce the expression of adhesion molecules on endothelial cells over time as well as blocking endothelial cell adhesion molecule 'outside-in' signals that are involved in leucocyte diapedesis.

Activation of NADPH oxidase in neutrophils is mediated in part by Rac1 [17]. In addition to the function of Rac1 in the formation of the active NADPH oxidase complex, it is reported to play a role in fibroblast and macrophage plasma membrane ruffling [48,49]. Rac1 may interact directly with the cytoskeleton during membrane ruffling, since Rac1 binds to the cytoskeletal protein tubulin and to POR1 (partner of Rac 1), a protein involved in membrane ruffling [50,51]. We show in the present study that VCAM-1 activates Rac1 within 30 s, and that this begins to decline after 2 min. Recently, van Wetering et al. [34] reported that VCAM-1 activated Rac1 in cytokine-stimulated HUVECs, but their earliest time point was 5 min. In addition, we report, using cells with stable transfection of dominant negative Rac1, that Rac1 is required for the activation by VCAM-1 of NADPH oxidase for production of low levels of ROS. The ROS generated by VCAM-1 stimulation then in turn modulate endothelial cell actin cytoskeletal structure [12]. In agreement with these data, van Wetering et al. [52] demonstrated that a membrane-permeant constitutively active Rac1 stimulated ROS production and actin stress fibre formation in endothelial cells. They also reported that this effect of active Rac1 on stress fibre formation was blocked by scavenging ROS and was mimicked by high levels of ROS (1 mM H₂O₂) [52]. Our data with dominant negative Rac1 stable transfectants are consistent with the recent report indicating that a membrane-permeant Rac1 peptide blocks VCAM-1-stimulated ROS production and intercellular gap formation [34]. We and others have also reported that scavenging ROS block VCAM-1-stimulated actin cytoskeletal rearrangements [12,34]. Oxygen metabolites have been shown to modulate actin structure. Catalase scavenging of H₂O₂ blocks fetal-calf-serum-stimulated mesangial cell contraction and production of ROS [53]. Furthermore, re-addition of oxygen to post-hypoxic cultured aortic endothelial cells induces reorganization of actin into ruffles, and this actin change is blocked by overexpression of superoxide dismutase, which scavenges superoxide [54]. Thus, during VCAM-1 signalling, Rac1 functions upstream of NADPH oxidase activity for the modulation of actin structure.

In summary, VCAM-1 stimulates intracellular calcium release, calcium channels and Rac1 activation, which are required for the activation of NADPH oxidase activity by VCAM-1. This NADPH oxidase activity generates low levels of ROS (0.5–1 μ M) [20] that induce actin restructuring for endothelial cell shape changes that occur during lymphocyte transendothelial migration [12]. This evidence contributes to our understanding of signalling by VCAM-1, a molecule that is important in diseases such as atherosclerosis, transplantation and asthma [1,3,4].

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