Involvement of Native TRPC3 Proteins in ATP-Dependent Expression of VCAM-1 and Monocyte Adherence in Coronary Artery Endothelial Cells

Kathryn Smedlund, Guillermo Vazquez

**Background**—Vascular cell adhesion molecule-1 (VCAM-1) is critical in monocyte recruitment to the endothelium, a key event in development of atherosclerotic lesions. Stimulation of human coronary artery endothelial cells (HCAECs) with ATP positively modulates VCAM-1 expression and function through a mechanism involving Ca²⁺ signaling. We here examined the role of Ca²⁺ influx and native TRPC3 channels in that mechanism.

**Methods and Results**—Omission of extracellular Ca²⁺ or pretreatment of cells with channel blockers markedly reduced ATP-induced VCAM-1 and monocyte adhesion. Using a siRNA strategy and real-time fluorescence, we found that native TRPC3 proteins contribute to constitutive and ATP-regulated Ca²⁺ influx. ATP-dependent upregulation of VCAM-1 was accompanied by an increase in basal cation entry and TRPC3 expression. Notably, TRPC3 knock-down resulted in a dramatic reduction of ATP-induced VCAM-1 and monocyte adhesion.

**Conclusions**—These findings indicate that in HCAECs, native TRPC3 proteins form channels that contribute to constitutive and ATP-dependent Ca²⁺ influx, and that TRPC3 expression and function are fundamental to support VCAM-1 expression and monocyte binding. This is the first evidence to date relating native TRPC3 proteins with regulated expression of cell adhesion molecules in coronary endothelium, and suggests a potential pathophysiological role of TRPC3 in coronary artery disease. (Arterioscler Thromb Vasc Biol. 2008;28:2049-2055)

**Key Words:** TRPC3 ▪ VCAM-1 ▪ monocyte recruitment ▪ Ca²⁺ influx ▪ atherogenesis

Recruitment of circulating monocytes to the arterial intima is a crucial event in initiation, progression, and fate of the atherosclerotic lesion. Indeed, monocyte infiltration in the subintima is observed early in atherogenesis, and also at more advanced stages, when plaque infiltration and neovascularization may occur.¹,² At the molecular level, monocyte adhesion to the vascular wall is secured by the interaction between the integrin αβ₁ (Very Late Antigen 4; CD49d/CD29) expressed on the monocyte and vascular cell adhesion molecule 1 (VCAM-1, CD106) on the endothelial cell.³ Next, VCAM-1–dependent signaling drives transendothelial migration of the bound monocyte. VCAM-1 is virtually absent in resting endothelium, but its expression is rapidly upregulated in response to proinflammatory and proatherogenic stimuli, particularly in vascular areas prone to lesion development.⁴ In vascular endothelium, nucleotides (ie, ATP, UTP) released to the extracellular milieu in response to ischemia, hypoxia, chemical, or mechanical stress are known to exert a strong proinflammatory effect (reviewed in⁵). For instance, ATP stimulates adhesion of neutrophils to pulmonary artery endothelium⁶ and promotes release of inflammatory mediators such as interleukin (IL)-6 and monocyte chemoattractant protein-1 in dermal microvascular endothelium.⁷ In human coronary artery endothelial cells (HCAECs) ATP induces expression of VCAM-1 and monocyte adhesion through stimulation of P2Y₁ receptors,⁸ in line with its effect in an in vivo model of neointima hyperplasia.⁹ This effect is specific for VCAM-1, as other cell adhesion molecules such as intercellular cell adhesion molecule-1 are not affected.¹⁰ The underlying signaling, although not fully defined, is known to involve transactivation of VEGF receptor (VEGFR) type 2 and stimulation of the small GTPase Rac1.¹¹ As is the case for several inflammatory mediators acting on vascular beds other than the coronary circulation,¹²,¹³ changes in intracellular Ca²⁺ levels associated to Ca²⁺ release from internal stores also seem to contribute to ATP-induced VCAM-1 in HCAECs.¹⁴,¹⁵ However, despite that in these cells stimulation of P2Y₂ receptors promotes a robust Ca²⁺ influx, the specific role of Ca²⁺ entry in regulation of VCAM-1 has not been examined. In pilot studies we found that HCAECs express all members of the Canonical Transient Receptor Potential (TRPC) family of channel forming proteins (TRPC1-7, except TRPC2, a pseudogene in humans¹⁶) and that TRPC3 forms, or is part of, endogenous...
in the presence or absence of channel blockers (Gd: Gd³⁺, 10 μmol/L; FFA: flufenamic acid, 50 μmol/L; SKF: SKF96365, 30 μmol/L; verapamil, 50 μmol/L) were processed for ELISA detection of surface VCAM-1. *P<0.01; **P<0.02; ***P<0.005, not quite significant; ns: not significantly different. Neither cell viability nor pH of the medium was altered by the channel blockers at these concentrations.

B, HCAECs were treated with ATP (100 μmol/L) or TNFα (10 ng/mL) for 3 hours before evaluation of monocyte adhesion. When indicated, cells were incubated with 10 μg/mL anti–VCAM-1 (VCAM-1-Ab, clone E-10, Santa Cruz) or 10 μg/mL anti-VEGFR1 (VEGFR1-Ab, clone RR9S, Santa Cruz) antibodies 45 minutes before addition of monocytes. *P<0.03 respect to control; **P<0.05 respect to control and TNFα alone; ***P<0.06, not quite significant respect to control, and P<0.04 respect to ATP alone.

**Methods**

**Cells and Transfections**

HCAECs (Lonzon, Calif) were grown in endothelial basal medium (EBM-2) supplemented with endothelial growth factors and 5% fetal bovine serum (FBS) at 37°C under humidified air (5% CO₂), and used between passages 2 to 10. U937 human monocytic cells (ATCC, Va) were grown in RPMI containing 10% FBS, TRPC3 siRNA (100 nmol/L; Dharmacon) or nonspecific oligonucleotides were delivered to cells with Lipofectamine2000 (Invitrogen) and counted (3 fields per well, triplicates/condition). In siRNA experiments, transfected HCAECs were plated onto 24-well plates for 48 hours and then processed as above.

**Cell Lysis and Immunoblotting**

Cells (~80% confluence) were made quiescent by replacing growth medium with EBM-2 (10 mmol/L glucose, no serum or growth factors) during 24 hours and processed for SDS-PAGE and immunoblotting as in.**15** Proteins were separated in 10% acrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-VCAM-1 (clone E-10, Santa Cruz), antibodies against TRPC1. 3 to 6 (Aiolone Labs), anti-TRPC7 (kindly provided by Dr W. Schilling, Case Western University School of Medicine), or anti-beta actin (Millipore). After incubation with secondary antibodies, immunoreactive bands were visualized by ECL (Amersham), quantified by densitometry within the linear range of the film, and their values normalized against those for β-actin.

**Cell ELISA**

HCAECs grown to confluence in 96-well plates were made quiescent as described above. After the indicated treatments cells were fixed in 0.5% glutaraldehyde, nonspecific sites blocked with 0.5% bovine serum albumin, and then incubated (1 hour, 37°C) with VCAM-1 monocolonal antibody (R&D Systems) and peroxidase-conjugated antiamouse antibody (Amersham). Peroxidase reaction was performed with 3,3′,5,5′-Tetramethylbenzidine (Sigma) and stopped with 2 N HCl within the linear range of color development (10 to 15 minutes). Cell surface VCAM-1 was estimated as optical density at 450 nm after background subtraction (OD. in the absence of primary antibody).

**Monocyte Adhesion**

HCAECs grown to confluence in 24-well plates were made quiescent as described above. After indicated treatments calcein-loaded U937 cells were added (50 000 per well) and incubation proceeded for 45 minutes at 37°C. After washes with PBS, bound monocytes were counted (3 fields per well, triplicates/condition). In siRNA experiments, transfected HCAECs were plated onto 24-well plates for 48 hours and then processed as above.

**Real-Time Fluorescence**

Coverslip-plated cells loaded with the Ca²⁺-sensitive dye Fura-2 were used to monitor real-time fluorescence changes of intracellular Ca²⁺ or Ba²⁺ on multiple cells with a charge-coupled device (CCD) camera-based imaging system (Intracellular Imaging Inc) as previously described.**15** Measurements were performed at room temperature and treatment conditions were in HEPES-buffered saline solution (HBSS) containing (in mmol/L): 140 NaCl, 4.7 KCl, 1 MgCl₂, 0.5% glutaraldehyde, nonspecific sites blocked with 0.5% bovine serum albumin, and then incubated (1 hour, 37°C) with VCAM-1 monocolonal antibody (R&D Systems) and peroxidase-conjugated antimouse antibody (Amersham). Peroxidase reaction was performed with 3,3′,5,5′-Tetramethylbenzidine (Sigma) and stopped with 2 N HCl within the linear range of color development (10 to 15 minutes). Cell surface VCAM-1 was estimated as optical density at 450 nm after background subtraction (OD. in the absence of primary antibody).

**Statistical Analysis**

Means of cytosolic Ca²⁺, rates of Ca²⁺/Ba²⁺ entry, or densitometric values were compared using a 2-tailed t test for two means, using Graph Pad InStat version 3.00 for Windows 95 (Graph Pad Software). Averaged results are from 3 to 5 independent experiments.

**Results**

Treatment of HCAECs with ATP (100 μmol/L) or tumor necrosis factor (TNF) α (10 ng/mL) induced a significant

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**Figure 1.** A, HCAECs treated with ATP (100 μmol/L) or TNFα (10 ng/mL) for 3 hours in the presence (+Ca²⁺) or absence (−Ca²⁺) of extracellular Ca²⁺, or ATP only (100 μmol/L, 3 hour) as described above. After the indicated treatments cells were fixed in HBSS containing (in mmol/L): 140 NaCl, 4.7 KCl, 1 MgCl₂, 0.5% glutaraldehyde, nonspecific sites blocked with 0.5% bovine serum albumin, and then incubated (1 hour, 37°C) with VCAM-1 monocolonal antibody (R&D Systems) and peroxidase-conjugated antiamouse antibody (Amersham). Peroxidase reaction was performed with 3,3′,5,5′-Tetramethylbenzidine (Sigma) and stopped with 2 N HCl within the linear range of color development (10 to 15 minutes). Cell surface VCAM-1 was estimated as optical density at 450 nm after background subtraction (OD. in the absence of primary antibody).

B, HCAECs were treated with ATP (100 μmol/L) or TNFα (10 ng/mL) for 3 hours before evaluation of monocyte adhesion. When indicated, cells were incubated with 10 μg/mL anti–VCAM-1 (VCAM-1-Ab, clone E-10, Santa Cruz) or 10 μg/mL anti-VEGFR1 (VEGFR1-Ab, clone RR9S, Santa Cruz) antibodies 45 minutes before addition of monocytes. *P<0.03 respect to control; **P<0.05 respect to control and TNFα alone; ***P<0.06, not quite significant respect to control, and P<0.04 respect to ATP alone.
increase in the amount of plasma membrane, or pathophysiologically relevant VCAM-1, as evaluated by cell ELISA (Figure 1A). VCAM-1 levels increased as early as 3 hours after treatment and started to decline by 16 to 24 hours (not shown). The effects of ATP and TNFα were also evident in total VCAM-1 protein levels, as evaluated by immunoblot analysis of whole-cell lysates (Figure 1A) and were also evident in total VCAM-1 protein levels, as evaluated by immunoblot analysis of whole-cell lysates (Figure 1A) and were not involved in adhesion,9 did not affect the extracellular domain of VCAM-1 (clone E-10, Santa Cruz) markedly reduced the binding of U937 monocytes, evidencing the contribution of VCAM-1 to the adhesion process (Figure 1B). Blocking VEGFR1 (Flt-1), expressed in typical biphasic Ca2+-response composed by a transient increase in cytosolic Ca2+ attributable to inositol triphosphate (IP3)-induced Ca2+ release from internal stores, which is followed by a robust Ca2+-influx phase (14 and Figure 2A). Both phases operate simultaneously, as indicated by experiments in which cells were challenged with ATP in the presence of extracellular Ca2+. As shown in Figure 1A, VCAM-1 levels were markedly reduced when Ca2+-free was omitted in the bath. Whereas TNFα effect was partially reduced (≈30% to 40%), that of ATP was completely abolished. Alternatively, we tested the effect of various Ca2+- channel blockers on ATP-induced VCAM-1. The inorganic pore channel blocker gadolinium, the non-selective cation channel blocker SKF96365 and flufenamic acid, and the nondihydropyridine verapamil, all caused a significant reduction of VCAM-1 expression (Figure 1A) at concentrations that markedly reduced ATP-dependent Ca2+- influx (inhibition of peak Ca2+-influx was: 95±3% with 10 μmol/L gadolinium or 30 μmol/L SKF96365; 85±6% with 50 μmol/L flufenamic acid; 45±15% with 50 μmol/L verapamil; all reductions had at least \( P<0.05 \) respect to control, \( n=3 \) to 4). These chemically unrelated blockers were chosen on the basis of their ability to block a broad spectrum of Ca2+-permeable channels, which includes store-operated and nonstore-operated channels with different degrees of selectivity for Ca2+ (10 and references therein). Importantly, neither treatment with channel blockers nor transfection with siRNA oligonucleotides (see below) altered expression of P2Y2 receptor (not shown). In HCAECs, ATP induces a typical biphasic Ca2+-response composed by a transient increase in cytosolic Ca2+ attributable to inositol triphosphate (IP3)-induced Ca2+-release from internal stores, which is followed by a robust Ca2+-influx phase (14 and Figure 2A). Both phases operate simultaneously, as indicated by experiments in which cells were challenged with ATP in the presence of extracellular Ca2+. As shown in Figure 2A, dotted trace.). Neither Ca2+-release nor influx were altered by NF279 or MRS2179, P2X and P2Y, antagonists, respectively17 (peak Ca2+-release and influx were, respectively, 185±20 and 138±10 nmol/L, regardless of the absence or presence of NF279 or MRS2179; \( n=15 \) to 22 cells), suggesting that the Ca2+-response was mediated by P2Y2 receptors, as is the case for ATP-induced VCAM-1 and monocyte adhesion.7 Under basal conditions, ie, in the absence of ATP stimulation, Ca2+-influx was not detectable (Figure 2A, open circles). However,
the use of Ba\(^{2+}\) (10 mmol/L) as a surrogate for Ca\(^{2+}\) unmasked the existence of constitutive or nonregulated cation influx (Figure 2B, control basal). The inability to detect constitutive cation influx with 2 mmol/L Ca\(^{2+}\) in the bath likely reflects operation of a highly efficient Ca\(^{2+}\) buffering system. In line with this, when cells were exposed to higher Ca\(^{2+}\) gradients (10 mmol/L in the bath), a significant yet transient Ca\(^{2+}\) influx was observed (not shown). Ba\(^{2+}\) is not subject to the counteracting actions of such buffering systems, and enters the cell unidirectionally, magnifying any existing basal influx (discussed in 19).

HCAECs express message for all members of the TRPC family, namely, TRPC1, 3 to 7 (TRPC2, a pseudogene in humans, is not present) and we confirmed expression at the protein level by immunoblot analysis of cell lysates (14 and Figure 2C). Among all TRPC proteins, TRPC3 forms channels endowed with significant constitutive activity. Using a siRNA approach we examined whether native TRPC3 contributed to constitutive cation influx in HCAECs. The results shown in Figure 2B (+TRPC3 siRNA, basal) indicate that indeed that is the case, as nonregulated Ba\(^{2+}\) influx was completely suppressed in cells transfected with siRNA oligonucleotides specific for TRPC3. Knock-down of TRPC3 also caused a significant reduction in both initial rate (2- to 3-fold decrease) and magnitude (≈50% reduction at peak) of ATP-induced Ca\(^{2+}\) influx (Figure 2A, open triangles) suggesting that TRPC3 is also an important component of receptor-regulated cation entry. Notably, ATP-dependent upregulation of VCAM-1 was accompanied by a gain in basal cation entry, as evidenced by a more than 2-fold increase in the rate of constitutive Ba\(^{2+}\) influx (Figure 2B, control+ATP). This Ba\(^{2+}\) influx remained unchanged in the presence of the phospholipase C inhibitor U73122 (not shown), indicating it was genuine nonregulated receptor-independent cation influx. Remarkably, this was correlated with a significant increase in TRPC3 protein levels after 3-hour treatment with ATP (Figure 3). Again, constitutive influx was absent if TRPC3 was knocked-down before treatment with ATP (Figure 2B, ATP+TRPC3 siRNA). No change was observed under these conditions in any of the other TRPC proteins expressed in HCAECs (not shown). In addition, the siRNA protocol targeted TRPC3 in an effective and specific manner, as protein expression levels of TRPC7, a structurally close relative of TRPC3, or the more distantly related member of the TRPC family, TRPC1, were not altered (Figure 2D).

Because Ca\(^{2+}\) influx was necessary for ATP-induced VCAM-1 and TRPC3 contributed to ATP-regulated Ca\(^{2+}\) influx, we next examined whether TRPC3 was part of the mechanism underlying ATP-regulated VCAM-1 expression and function. The experiments in Figure 4A show that knock-down of TRPC3 completely reduced ATP-induced VCAM-1. Of importance, VCAM-1 is not the sole cell adhesion molecule mediating monocyte adhesion, whereas Ca\(^{2+}\) influx is a critical component of the signaling associated to monocyte adhesion and migration, regardless of the adhesion molecules involved. Thus, we examined to what extent Ca\(^{2+}\) influx or TRPC3 were required for monocyte adhesion to HCAECs. Cells were exposed to ATP in the presence or absence of extracellular added Ca\(^{2+}\) or pretreated with channel blockers, and monocyte adhesion was evaluated as described in Methods. Alternatively, HCAECs were transfected with TRPC3 siRNA (100 nmol/L) and 48 hours later processed for immunodetection of TRPC3. Bars show average normalized values of densitometric analysis of 5 experiments, expressed as fold induction over control (vehicle-treated cells). *P<0.04.

**Figure 3.** HCAECs were treated with ATP (100 μmol/L, 3 hours) and processed for immunodetection of TRPC3. Bars show average normalized values of densitometric analysis of 5 experiments, expressed as fold induction over control (vehicle-treated cells). *P<0.04.

**Discussion**

The importance of Ca\(^{2+}\) signaling in regulated expression of VCAM-1 has been appreciated in previous studies. For example, changes in intracellular Ca\(^{2+}\) associated to Ca\(^{2+}\) release from internal stores have been linked to the ability of Substance P and β₂-microglobulin to induce VCAM-1 in microvascular endothelium and synovial fibroblasts, respectively. In HCAECs, Ca\(^{2+}\) mobilization has been related to the mechanism by which lipoprotein A and ATP promote VCAM-1 expression. Nevertheless, the specific role of Ca\(^{2+}\) influx has not been directly examined. Besides, in most instances VCAM-1 expression was evaluated under conditions of strong cytosolic Ca\(^{2+}\) buffering, which may prevent a contribution from Ca\(^{2+}\) entry if Ca\(^{2+}\) microdomains at the channel mouth are perturbed. Here we addressed the role of Ca\(^{2+}\) influx in ATP-dependent regulation of VCAM-1 in HCAECs. Several important conclusions can be derived from the present findings. That Ca\(^{2+}\) influx contributes to the signaling underlying ATP-induced VCAM-1 was first suggested by the observation that maneuvers that prevent Ca\(^{2+}\) entry into HCAEC significantly impaired VCAM-1 expression. This was evident not only on the amount of total cellular VCAM-1 protein, but most importantly on the levels of
alk cerebral microvascular endothelial cells (HCAECs). The latter are involved in the development of atherosclerotic plaques and play a role in the recruitment of monocytes and other leukocytes. TRPC3 is a member of the transient receptor potential (TRP) family of ion channels, which are involved in various cellular processes including calcium influx, cell adhesion, and cytokine production. The study by Smedlund and Vazquez investigated the role of TRPC3 in the expression of VCAM-1, a protein involved in monocyte adhesion, in HCAECs.

The authors found that ATP treatment increased VCAM-1 expression, which was partly mediated by TRPC3. They also observed that TRPC3 expression correlated with augmented constitutive cation influx, suggesting that TRPC3 contributes to constitutive activity of the channel. Furthermore, knock-down of TRPC3 expression by siRNA significantly reduced ATP-induced VCAM-1 expression, indicating the importance of TRPC3 in this process.

The study highlights the role of TRPC3 in the regulation of VCAM-1 expression and monocyte adhesion, which is crucial for understanding the pathophysiology of atherosclerosis. The findings also suggest potential therapeutic targets for the treatment of this disease.

Graphical abstract:

**Figure 4.** A, HCAECs transfected with nonspecific oligonucleotides (control) or TRPC3 siRNA were treated with ATP (100 μmol/L, 3 hours) and processed for ELISA detection of surface VCAM-1. *P<0.01. B, HCAECs were treated with ATP (100 μmol/L, 3 hour) in the presence or absence of extracellular Ca2+, or channel blockers (see legend to Figure 1A for details) or transfected with nonspecific oligonucleotides (nso) or TRPC3 siRNA before ATP treatment, and then monocyte adhesion was evaluated. *P<0.03 respect to control; **P<0.02 respect to ATP treatment in normal conditions, P<0.05 respect to their corresponding control; ns: not significantly different.

The study by Smedlund and Vazquez emphasizes the role of TRPC3 in the expression of VCAM-1 in HCAECs, which is crucial for understanding the pathophysiology of atherosclerosis. The findings also suggest potential therapeutic targets for the treatment of this disease.
and function, it must be in combination with TRPC3. Otherwise, homo-tetramers made of TRPC proteins other than TRPC3 would be expected to behave independently, and their contribution to VCAM-1 expression should remain, even after knocking-down TRPC3; nevertheless, TRPC3 siRNA completely abrogated ATP-induced VCAM-1 and monocyte binding.

TRPC channels are now recognized among the most important Ca^{2+}-permeable cation channels in vascular endothelium physiology. In addition, it is becoming evident that they are critical players in cardiovascular disease. For instance, TRPC1, 4, and 6 participate in regulation of vascular tone and thus play a role in hypertension. Other TRPC channels, such as TRPC1, 4, and 6 participate in regulation of vascular endothelial cells and cellular and molecular events that are crucial in development of the atherosclerotic lesion. Advances on elucidating molecular and cellular components involved in lesion formation and progression, such as VCAM-1 and its role in monocyte recruitment, have been enthusiastically received in the field as promising new opportunities to develop antiinflammatory therapies for atherosclerosis. Therefore, identifying new players within the signaling underlying VCAM-1 expression and function is imperative to develop alternative therapeutic targets for effective treatment of this disease. Within that context, our studies warrant further in vitro and in vivo studies to determine the relevance of TRPC3 in development and progression of coronary artery disease.

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Disclosures
None.

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