Cholesterol Depletion Impairs Vascular Reactivity to Endothelin-1 by Reducing Store-Operated Ca\(^{2+}\) Entry Dependent on TRPC1

Andreas Bergdahl, Maria F. Gomez, Karl Dreja, Shang-Zhong Xu, Mikael Adner, David J. Beech, Jonas Broman, Per Hellstrand, Karl Swärd

Abstract—The reactivity of the vascular wall to endothelin-1 (ET-1) is influenced by cholesterol, which is of possible importance for the progression of atherosclerosis. To elucidate signaling steps affected, the cholesterol acceptor methyl-\(\beta\)-cyclodextrin (m\(\beta\)cd, 10 mmol/L) was used to manipulate membrane cholesterol and disrupt caveolae in intact rat arteries. In endothelium-denuded caudal artery, contractile responsiveness to 10 nmol/L ET-1 (mediated by the ET\(_A\) receptor) was reduced by m\(\beta\)cd and increased by cholesterol. Neither ligand binding nor colocalization of ET\(_A\) and caveolin-1 was affected by m\(\beta\)cd. Ca\(^{2+}\) influx via store-operated channels after depletion of intracellular Ca\(^{2+}\) stores was reduced in m\(\beta\)cd-treated arterial, as shown by Mn\(^{2+}\) quench rate and intracellular [Ca\(^{2+}\)] response. Expression of TRPC1, 3, and 6 was detected by reverse transcriptase–polymerase chain reaction, and colocalization of TRPC1 with caveolin-1 was reduced by m\(\beta\)cd, as seen by immunofluorescence. Part of the contractile response to ET-1 was inhibited by Ni\(^{2+}\) (0.5 mmol/L) and by a TRPC1 blocking antibody. In the basilar artery, exhibiting less store-operated channel activity than the caudal artery, ET-1–induced contractions were insensitive to the TRPC1 blocking antibody and to m\(\beta\)cd. Increased store-operated channel activity in basilar arteries after organ culture correlated with increased sensitivity of ET-1 contraction to m\(\beta\)cd. These results suggest that cholesterol influences vascular reactivity to ET-1 by affecting the caveolar localization of TRPC1. (Circ Res. 2003;93:839-847.)

Key Words: arterial smooth muscle ■ methyl-\(\beta\)-cyclodextrin ■ caveolae ■ endothelin ■ store-operated Ca\(^{2+}\) channels

Hypercholesterolemia increases reactivity to endothelin-1 (ET-1) in experimental animals and humans.\(^1\)\(^-\)\(^4\) This has been pointed out as one possible factor in the progression of atherosclerosis.\(^5\)\(^-\)\(^7\) The mechanism of action has not been elucidated, although both endothelial dysfunction and altered smooth muscle reactivity have been proposed.\(^5\) Lipoprotein particles may directly influence endothelial membrane–associated endothelial NO synthase activity by interfering with cholesterol-rich domains referred to as caveolae.\(^8\) Although these effects modulate the endothelial influence on vascular tone, less is known regarding direct effects of cholesterol on vascular smooth muscle functions.

Caveolae are 50- to 100-nm membrane invaginations that integrate many cellular receptor functions.\(^9\) For instance, ET\(_A\) receptors expressed in COS cells colocalize with the caveolae-associated protein caveolin.\(^10\)\(^,\)\(^11\) The caveolar structure is disrupted after depletion of cholesterol with cyclodextrins,\(^12\) and this correlates with a decreased contractility to ET-1, but not to depolarization or \(\alpha_1\)-receptor stimulation, in endothelium-denuded rat caudal arteries.\(^13\) Cholesterol might thus modulate the strength of caveolae-associated signaling, providing a basis for altered contractility in response to ET-1.

Activation of the ET\(_A\) receptor stimulates Ca\(^{2+}\) influx over the plasma membrane primarily via non–voltage-dependent cation channels, including a store-operated inflow, as revealed by pharmacological analysis in rat aorta.\(^14\) In rabbit cerebral arterioles, ET-1 was found to activate receptor-operated non–voltage-dependent Ca\(^{2+}\) channels, whereas no evidence was found for potentiation by ET-1 of Ca\(^{2+}\) inflow stimulated by depletion of intracellular Ca\(^{2+}\) stores.\(^15\) Definition of the relative roles of receptor-operated channels (ROCs) versus store-operated channels (SOCs) in ET-1 responses thus depends on the tissue and the selectivity of available tools.

The functionally defined ROC and SOC in smooth muscle may be closely related, involving proteins coded by genes homologous to the transient receptor potential (\(\text{trp}\)) genes in Drosophila.\(^16\) The mammalian \(\text{trp}\) counterparts constitute a large family of ion channels,\(^17\) and the TRPC proteins (TRPC 1 to 7) have been associated with store- and receptor-operated...
Ca²⁺ entry. Evidence for TRPC1 as a membrane-spanning subunit of SOC in vascular smooth muscle cells has been obtained using a specific blocking antibody. Among potential ROC-related TRPC isoforms, activation of TRPC6 by α-adrenergic stimulation, possibly mediated by diacyl glycerol, has been reported. Activation of other TRPC isoforms has been variously ascribed to store depletion and binding of diacyl glycerol or inositol-trisphosphate. Although information is rapidly accumulating, much of it is based on heterologously expressed channels, and there is still insufficient knowledge about the molecular composition of endogenous channels.

Given the association of ET-1 signaling with SOC and ROC activity on the one hand and its dependence on membrane cholesterol on the other, it is possible that the effect of cholesterol is associated with an influence on ion channel activity involving TRPC proteins. We have therefore investigated the effects of cholesterol extraction on ET-1 responses in vascular preparations showing different levels of SOC activity. We demonstrate that vascular SOC activity is sensitive to cholesterol depletion and that inhibition of ET-1 responses by cholesterol depletion may be accounted for by reduced caveolar targeting of TRPC1.

Materials and Methods

Contractility Measurements
Female Sprague-Dawley rats (200 g; Mollegard, Copenhagen, Denmark) were killed by cervical dislocation, as approved by the Animal Ethics Committee, Lund University, and caudal and basilar arteries were removed. Segments were dissected in Ca²⁺-free PSS (see below), and endothelium was removed with a stainless steel thread. Vessels were mounted for force measurements, and single doses of ET-1 were applied. Concentration-response curves were assembled from arterial segments from the same animal run in parallel. Removing the endothelium did not affect the response to high K⁺ (HK) (60 mmol/L), which was 11.2 ± 1 versus 12.1 ± 1 mN/mm (P > 0.05, n = 8) for intact and denuded caudal arteries and 5.1 ± 0.6 versus 6.1 ± 1 mN/mm (P > 0.05, n = 6) for basilar arteries.

TRPC1 Blocking Antibody Incubation and Organ Culture
Caudal artery segments (1 mm) were incubated with the TRPC1 antibody T1E3 (1:500 in PSS) at 4°C overnight. Controls were treated with preimmune serum from the rabbit used to generate T1E3 (1:500, n = 13) or vehicle (0.15 mg/mL sodium azide in PSS, n = 13). Preimmune serum was without effect. After incubation, preparations were mounted in PSS and depolarized with 60 mmol/L HK until stable contractions were attained. ET-1 was added in the presence of T1E3, preimmune serum, or vehicle. Basilar arteries (2 mm) were treated identically, but the preimmune serum control was omitted. Arteries were cultured for 4 days under serum-free conditions.

Determination of [Ca²⁺]
[Ca²⁺], and Mn²⁺ quench rates were measured using Fura 2 (Molecular Probes) as described. [Ca²⁺] is reported as the ratio of Fura 2 fluorescence for excitation at 340 and 380 nm.

[¹²⁵]ET-1 Binding
Four denuded and segmented (5-mm) caudal arteries were divided equally into four Ellermann tubes containing 1 mL PSS. Tubes were incubated for 50 minutes at 37°C with or without methyl-β-cyclodextrin (mβcd). After washing, 100 mmol/L cold ET-1 was added to one mβcd-treated and one control tube, followed by addition after 30 minutes of 0.1 mmol/L [¹²⁵]ET-1 (2 μCi) to all tubes. After incubation at room temperature for 2 hours, pellets were washed in PSS (×4) and [¹²⁵]ET-1 binding was determined by γ-counting.

Reverse Transcriptase–Polymerase Chain Reaction Detection of TRPC Isoforms
Arteries were transferred to nuclease-free microfuge tubes, frozen in liquid nitrogen, and stored at −80°C. Total RNA (1.2 to 1.4 μg) prepared using Trizol reagent (Life Technologies) was DNase-treated and reverse-transcribed (RT) using oligo-dT primers and Sensiscript RT Kit (Qiagen). RT negative controls were prepared without Sensiscript. Polymerase chain reaction (PCR) reactions were performed using HotStarTaq (Qiagen) with specific primer pairs (see the online Table, available at http://www.circresaha.org) according to the following protocol: denaturation at 94°C for 12 minutes, 40 cycles of denaturation at 94°C for 1 minute, annealing at 54.5°C for 30 seconds, extension at 72°C for 2 minutes, and prolonged extension at 72°C for 10 minutes. Amplification products were electrophoresed on agarose gels and visualized by ethidium bromide staining.

Sucrose Density Fractionation and Western Blotting
Three to five caudal arteries per experiment were homogenized and ultracentrifuged on 5% to 45% discontinuous sucrose gradients, and 10×1 mL fractions were recovered. Proteins were precipitated with 10% trichloro-acetic acid, and pellets were washed with acetone resuspended in Laemmli sample buffer. Proteins were visualized by Western blotting using antibodies to Caveolin-1 (Cav-1) (6B6, a gift from Dr J. Vinten, University of Copenhagen, Denmark; 1 μg/mL) and TRPC1 (T1E3 1:500). 6B6 and T1E3 have been characterized. Peroxidase-conjugated secondary antibodies were used, and bands were detected by chemiluminescence (Pierce).

Immunoprecipitation
Denuded rat caudal arteries were homogenized and sonicated in IP buffer (20 mmol/L HEPES-NaOH [pH 7.5], 1% Triton X-100, 150 mmol/L NaCl, and protease inhibitor cocktail) followed by centrifugation at 16 000 rpm (20 minutes, 4°C). Two hundred micrograms of protein from the supernatant was used for immunoprecipitation with 2 μg of the appropriate antibody and 10 μg (30 μL) of prewashed and swelled antibody Protein A Sepharose CL-4 B (Sigma) or protein G beads (Pierce). The IP cocktail was agitated for 4 hours at 4°C, and pelleted antibody–protein A bead complexes were washed with 1 mL IP buffer (×3) and PBS (×1). Complexes were resuspended in 30 μL of PBS and 30 μL of 2% Laemmli sample buffer, and Western blotting was performed.

Immunohistochemical Visualization of ETₐ, TRPC1, and Cav-1
Arteries were incubated for 60 minutes at 37°C with or without 10 nmol/L mβcd followed by fixation in 4% formaldehyde in PBS (pH 7.4, 15 minutes). Vessels were embedded in Tissue-Tek (Sakura) and frozen. Transverse and longitudinal sections (10 μm), permeabilized with 0.2% Triton-X-100 in PBS for 10 minutes, were mounted and examined at ×100 magnification using a Zeiss LSM 510 confocal microscope. TRPC1 and ETₐ were detected by monitoring Cy5 fluorescence at 670 nm on excitation at 650 nm. Cav-1 was monitored at 520 nm on excitation at 492 nm. Multiple fields for each vessel were analyzed under blind conditions. Sequential images for Cy5 and Cy2 fluorescence were merged, and colocalization was calculated using the Zeiss LSM 510 Pascal Analysis software (version 3.2) or by superimposing three
Results

Cholesterol Modulates Reactivity to ET-1 in Rat Caudal Artery

Treatment of endothelium-denuded caudal arteries with the cholesterol acceptor mβcd (40 minutes), which extracts ≈20% of tissue cholesterol,13 reduced reactivity to ET-1 (10 mmol/L, Figures 1Aa and 1Ab). Replenishment of cholesterol using cholesterol-mβcd (2:10 mmol/L), which delivers cholesterol into cells, led to recovery of ET-1 contraction and subsequent potentiation relative to control (Figures 1Ab through 1Ae and 1B). Inhibition by mβcd was time-dependent, showing a significant reduction of ET-1 contraction already after 20 minutes (Figure 2A). Sixty-minute treatment was subsequently used as the standard protocol for cholesterol depletion. Figure 2B shows contractions elicited by ET-1 in a control and a cholesterol-depleted artery and in an artery that had been treated with cholesterol-mβcd without prior depletion, demonstrating that increased cholesterol amplifies ET-1 contractions. Compiled force data from experiments in Figure 2B are shown in Figure 2C. Thus, the response to ET-1 varies both as a function of incubation time with mβcd and as a function of the cholesterol saturation of mβcd.

Measurements of global intracellular Ca²⁺ ([Ca²⁺]i) revealed decreased responses to ET-1 after cholesterol depletion (Figure 2D). Expressed relative to HK responses, which are unaffected by mβcd,13 the elevation of [Ca²⁺]i during stimulation with ET-1 was 81±8% in control preparations and 33±1% after cholesterol depletion (P<0.005, n=3).

No Effect of Cholesterol Depletion on Ligand Binding or Localization of ET-1 Receptors

ET-1 induced vasoconstriction via binding to ET₄ or ET₃ receptors.20 To determine which is involved, the ET₄ selective ligand sarafotoxin6c was used. At concentrations that have been shown effective in other rat vascular preparations (10 nmol/L and 1 μmol/L), we were unable to generate force in either control or mβcd-treated endothelium-denuded arteries (n=4). The selective ET₃ blocker RF139317 (100 μmol/L) reduced contraction to 10 nmol/L ET-1 by 70%.
(11±5% versus 36±2% of HK, P<0.01, n=3), suggesting that ET, mediates contraction in response to ET-1. Muscle cell caveolae are reversibly disrupted by cholesterol depletion,13 and localization of ET, in caveolae has been demonstrated.10 We therefore investigated whether the number of available receptors or the ligand-receptor interaction might be affected by changes in cholesterol levels. Cholesterol extraction failed to affect the binding of [125I]ET-1 (Figure 3A) or the EC50 value (13±2 versus 12±1 nmol/L, Figure 3B), whereas the amplitude of contraction was reduced at all concentrations (Figure 3B).

The lack of effect of mβcd on ET-1 binding suggests that downstream signaling is disrupted. To test whether this might involve displacement of ET, from caveolae, we stained caudal artery sections with antibodies against ET, (red in Figure 3Cb) and the caveolar marker Cav-1 (green, Figure 3Cc). Colocalization (yellow, Figure 2Ca) was observed at or near the smooth muscle cell plasma membrane. Some membrane sections are only green, indicating that not all Cav-1-rich regions contain ET,. This was observed in transverse cell sections, especially at focal planes near the center of the cells (Figure 2Ca, white arrow), whereas ET,-rich regions were predominant near cell ends (Figure 2Ca, white arrowheads). Colocalization coefficients were not different between control and mβcd-treated arteries (0.76±0.02 and 0.63±0.03 versus 0.73±0.03 and 0.62±0.03 for ET, and Cav-1, respectively). Thus, although ET, receptors seem to be preferentially localized to caveolae in the caudal artery, this experiment does not indicate that cholesterol depletion uncouples ET-1 signaling by displacing receptors out of caveolae.

Cholesterol Extraction Reduces Store-Operated Ca2+ Entry and Activation

An alternative explanation involves effects on Ca2+ influx pathways that are activated on ET-1 stimulation. The contributions of different Ca2+-permeable channels to ET-1 responses vary between smooth muscle preparations.14,15 In the caudal artery, ET-1–induced contractions were significantly inhibited by verapamil (10 μmol/L) and also by Ni2+ (0.5 mmol/L). The combination of both of these inhibitors
totally abolished contraction in response to ET-1 (Figure 4A). Thus, ET-1 elicits a contraction partly dependent on Ca²⁺ influx via voltage-dependent Ca²⁺ channels (blocked by verapamil) and partly on nonselective ROC or SOC (blocked by Ni²⁺).

Because mβcd did not affect HK-induced Ca²⁺ elevation (Figure 2D) or contraction, we considered it less likely that cholesterol depletion influences activity of voltage-dependent Ca²⁺ channels and therefore focused on a possible effect on store-operated Ca²⁺ influx. Uptake of Ca²⁺ into the sarcoplasmic reticulum was irreversibly inhibited by 10 minutes of exposure to thapsigargin (25 μmol/L), and stores were subsequently depleted by caffeine (20 mmol/L) in Ca²⁺-free medium. Figure 4B shows Fura-2 recordings of [Ca²⁺] in readdition of Ca²⁺ (2.5 mmol/L) after store depletion. The rise in [Ca²⁺], is reduced after mβcd (P<0.05), suggesting an effect on store-operated influx. By measuring the rates of fluorescence quench by Mn²⁺ in Fura-2-loaded arteries, we confirmed that store-operated Ca²⁺ influx is inhibited by cholesterol extraction. Depletion of Ca²⁺ stores increased the quench rate 2-fold in control arteries but had no effect after mβcd treatment (Figure 4C). Transient contractions to ET-1 (100 nmol/L) in the absence of extracellular Ca²⁺ (100 μmol/L EGTA) were also reduced by mβcd (from 27±4% to 14±4% of HK, P<0.05, n=8), suggesting reduced Ca²⁺ release from intracellular stores.

**Cholesterol Extraction Reduces TRPC1 and Caveolin-1 Colocalization**

RT-PCR demonstrated the presence of transcripts for TRPC1, TRPC3, and TRPC6 in caudal artery (Figure 5A). Positive controls from brain (Figure 5B), testis (Figure 5C), and lung (Figure 5D) generated products with predicted sizes (see the online Table). TRPC1 partially copurified with the caveolar marker Cav-1 on sucrose density fractionation, as shown by Western blotting (Figure 5E). Immunoprecipitation of Cav-1 pulled down TRPC1 (Figure 5G), again supporting colocalization of these proteins.

Partial colocalization of TRPC1 (blue) and Cav-1 (green) was confirmed by immunofluorescence of TRPC1 and Cav-1 in caudal artery sections (Figure 6A). Analysis of the intensity profile along a line indicates lower degree of colocalization of TRPC1 and Cav-1 after mβcd (Figure 6B). Colocalization coefficients for control and treated arteries were 0.85±0.04 and 0.75±0.04 versus 0.57±0.03 and 0.61±0.03 for TRPC1 and Cav-1, respectively (P<0.05, n=5). In contrast to Cav-1 labeling, which is predominantly observed at or near the smooth muscle plasma membrane in both control and mβcd-treated arteries, a more diffuse TRPC1 staining pattern was found after cholesterol extraction, consistent with less TRPC1 labeling confined to the cell surface. The total number of peaks detected for each dye and mean pixel intensities were not affected, suggesting redistribution rather than decreased immunostaining to underlie the reduced colocalization.

Although our results support a role of cholesterol in store-operated Ca²⁺ entry, possibly involving TRPC1, we cannot rule out an effect of cholesterol extraction involving TRPC3 and TRPC6, which may form channels activated by diacyl glycerol. Using the membrane permeable diacyl glycerol analogue 1-oleoyl-2-acetyl-sn-glycerol (300 μmol/L), we were unable to elicit either contraction or [Ca²⁺] elevation in the presence of verapamil (not shown).

**Different Responses to Cholesterol Depletion Reflect Different Levels of SOC Activity**

The apparent displacement of TRPC1 from caveolae and the reduced store-operated Ca²⁺ influx suggest that the cholesterol sensitivity of the ET-1 response is associated with TRPC1 activity. To test this concept, we took advantage of our findings that the basilar artery exhibits lower SOC activity than the caudal artery but that a prominent increase occurs after organ culture. In contrast to the findings in the caudal artery (Figure 4C), the Mn²⁺ quench rate was not stimulated by store depletion in fresh basilar artery (108±9%, P>0.05, n=4). As in caudal arteries, a clear-cut effect of mβcd on basilar artery caveolae, with different degrees of flattening and dilation, was observed by electron microscopy (Figures 7A and 7B). Yet mβcd had no effect on the response to ET-1 (Figure 7C). Similarly, transient contractions in Ca²⁺-free medium, reflecting Ca²⁺-release from internal stores, were unaffected (43±6 versus 48±5% of HK).
The effects of various blockers on ET-1–induced contractions in caudal and basilar arteries are shown in Figure 7D. Although verapamil had similar effects in the two preparations, Ni²⁺ gave a significantly smaller relaxing effect in the basilar than in the caudal artery. In the presence of verapamil, inhibition of residual SOC-mediated contraction by Ni²⁺ or SKF-96365 was less efficient in the basilar artery. 2-APB and Gd³⁺, which block nonselective cation channels, had similar effects in both arteries.

Using an alternative approach to determine the contribution of TRPC1 activity to ET-1 contraction, caudal and basilar artery segments were incubated with the T1E3 antibody, as described in the Materials and Methods section. This antibody binds to the outer pore region of TRPC1 and blocks store-operated Ca²⁺ entry. T1E3 inhibited ET-1 contraction in the caudal artery by 40% (Figure 7E). In the basilar artery, T1E3 had no effect (Figure 7F).

**Increased Sensitivity to Cholesterol Depletion After Organ Culture**

The correlation between level of SOC activity and sensitivity to cholesterol depletion strengthens the evidence for the SOC component of the ET-1 response as the cholesterol-sensitive step. To rule out a qualitative difference in the coupling of receptor occupation to downstream signaling, we induced increased SOC activity in both caudal and basilar arteries by organ culture for 4 days. Store-operated Ca²⁺ entry is associated with a small force response in fresh caudal arteries, whereas no response is seen in fresh basilar arteries. After organ culture, the verapamil-resistant store-operated [Ca²⁺],...
elevation and its associated force response are increased in both vessels. As shown in Figures 8A and 8B, SOC-associated contractions were effectively reduced in cultured arteries by mβcd. In contrast to fresh basilar arteries, mβcd treatment significantly reduced ET-1 contraction after culture and an increased relative sensitivity to Ni²⁺ was observed (Figure 8C).

**Discussion**

This study demonstrates an acute influence of cellular cholesterol on arterial reactivity to ET-1. In endothelium-denuded caudal artery, cholesterol extraction with mβcd lowered reactivity to ET-1, correlating with reduced store-operated Ca²⁺ entry. In denuded basilar arteries where Ca²⁺ entry is little affected by store depletion, there was no effect of mβcd on the response to ET-1. Moreover, organ culture, which increases store-operated Ca²⁺ entry several-fold, conferred sensitivity of ET-1 contraction to mβcd. Cholesterol depletion thus diminishes the contractile effect to ET-1 by a mechanism that seems to involve store-operated channels. The different reactions of the caudal and basilar arteries are probably explained by a lower functional expression of channels in the basilar artery. This diversity, although possibly important for selective responses in different vascular beds, is not attributable to fundamentally different signaling mechanisms. Thus, the cholesterol sensitivity of the force response associated with store-operated Ca²⁺ entry correlates well with that of the ET-1 response. This applies both to a comparison between the two arteries studied here and to the plasticity apparent during organ culture.

The verapamil-resistant component of ET-1–induced contraction in the tail artery, which was sensitive to Ni²⁺ and SKF-96365, may in part represent SOC activity. In accordance with this hypothesis, our data demonstrate quantitatively greater effects of Ni²⁺ and SKF-96365 on caudal than on cerebral artery contraction. Capacitative Ca²⁺ entry in the caudal artery is associated with a small force response (5% to 10% of HK responses), despite elevating [Ca²⁺], to nearly 60% of the HK response. The response to ET-1 involves Ca²⁺ sensitization, which may increase the contractile effect of SOC activation. A significant SOC-dependent component in the ET-1 response in the caudal artery is additionally supported by the fact that the TRPC1 antibody inhibited force by 40%. In contrast, no effect was observed in the basilar artery, again indicating that the store-operated component of Ca²⁺ influx in this vessel is small.

TRPC1, TRPC3, and TRPC6 were expressed in denuded caudal artery. Of these, TRPC1 has been shown to mediate store-operated Ca²⁺ influx in vascular smooth muscle, whereas TRPC3 and TRPC6 are activated by diacyl glycerol. TRPC6 may be associated with receptor-operated Ca²⁺ entry in native vascular smooth muscle and has been implicated in myogenic tone in cerebral arteries, but the functional role of TRPC3 in this tissue is unclear. Heterologous expression experiments have suggested that TRPC3 may form either receptor- or store-operated channels.

TRPC1 coimmunoprecipitated with Cav-1 and TRPC1 was found in Cav-1–containing fractions of caudal artery homogenates but also in membrane domains of higher density. Immunofluorescence of tissue sections similarly demonstrated a partial colocalization of TRPC1 and Cav-1, which was sensitive to cholesterol depletion. This suggests that TRPC1 is partially associated with rafts/caveolae and that this distribution is altered when these structures are disrupted after cholesterol depletion. It remains to be determined whether increased cholesterol can increase the association of TRPC1 with caveolae. In nonvascular cell types, TRPC1, TRPC3, and TRPC4 have previously been associated with caveolae or lipid rafts, whereas no information on the localization of TRPC6 or its dependence on membrane cholesterol is available.

Multiple lines of biochemical evidence for partial localization of TRPC1 in caveolin-containing raft domains in submandibular gland cells were provided by Lockwich et al. This included light buoyant density of TRPC1, resistance to extraction by Triton X-100 at 4°C, coimmunoprecipitation with caveolin, and sensitivity to cycloheximide treatment. Similar to our results, TRPC1 was partially associated with rafts, and yet complete inhibition of SOC was observed after mβcd treatment. The basis of this apparent inconsistency is not presently known but may involve effects on the activation mechanisms as well as the SOC channel itself. Indeed, transient contractions in Ca²⁺-free medium were reduced by mβcd in caudal but not basilar artery, suggesting effects on
Ca\textsuperscript{2+} release, ie, reflecting a possible role of SOC in store refilling. Although this would contribute to the effect of m\textgreek{j}cd on ET-1 responses, it does not account for all of the effect on Ca\textsuperscript{2+} inflow, as shown by the results using thapsigargin. Because TRPC3 and TRPC6 are expressed in caudal artery, a possible association of these channels with lipid rafts/caveole and ET-1 signaling might also contribute to cholesterol sensitivity.

In summary, the present study demonstrates a direct effect of cholesterol depletion on reactivity to ET-1, correlating...
with inhibition of SOC activity and redistribution of TRPC1 in the membrane. The dependence on SOC activity seems to determine sensitivity of ET-1 responses to cholesterol and may underlie altered sensitivity to ET-1 in dyslipidemia.

Acknowledgments

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Table I. Oligonucleotide sequences of the primers used for RT-PCR

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The specificity of the TRPC primers has been verified by sequencing\textsuperscript{1-3} and expected sizes of amplicons are given in the right column (bp = basepairs).

References

