Activation of TRPV1 reduces vascular lipid accumulation and attenuates atherosclerosis

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Abstract

Activation of transient receptor potential vanilloid type-1 (TRPV1) channels may affect lipid storage and the cellular inflammatory response. Now, we tested the hypothesis that activation of TRPV1 channels attenuates atherosclerosis in apolipoprotein E knockout mice (ApoE−/−) but not ApoE−/−/TRPV1−/− double knockout mice on a high-fat diet. Both TRPV1 mRNA and protein expression were identified in vascular smooth muscle cells (VSMC) and in aorta from C57BL/6J mice using RT–PCR, immunoblotting, and immunohistochemistry. In vitro, activation of TRPV1 by the specific agonists capsaicin and resiniferatoxin dose-dependently increased cytosolic calcium and significantly reduced the accumulation of lipids in VSMC from C57BL/6J mice but not from TRPV1−/− mice. TRPV1 activation increased ATP-binding cassette transporter A1 (ABCA1) expression and reduced low-density lipoprotein-related protein 1 (LRP1) expression in VSMC by calcium-dependent and calcineurin- and protein kinase A-dependent mechanisms. These results showed increased cellular cholesterol efflux and reduced cholesterol uptake. In vivo, long-term activation of TRPV1 by capsaicin for 24 weeks increased ABCA1 and reduced LRP1 expression in aorta from ApoE−/− mice on a high-fat diet. Long-term activation of TRPV1 significantly reduced lipid storage and atherosclerotic lesions in the aortic sinus and in the thoracoabdominal aorta from ApoE−/− mice but not from ApoE−/−/TRPV1−/− mice on a high-fat diet. These findings indicated that TRPV1 activation ameliorates high-fat diet-induced atherosclerosis.

Keywords

Transient receptor potential vanilloid type-1 • Atherosclerosis • Capsaicin • Vascular smooth muscle cells

1. Introduction

Atherosclerosis is considered to be an inflammatory process consisting largely of the accumulation of lipids within the artery wall.1,2 Vascular smooth muscle cells (VSMC) have been demonstrated to express a variety of cholesterol uptake receptors and reverse cholesterol transporters, including low-density lipoprotein (LDL) receptor, LDL receptor-related protein 1 (LRP1), and ATP-binding cassette transporter A1 (ABCA1).2,5 These studies suggest that lipid accumulation in VSMC contributes to atherosclerosis development. Recent studies showed that transient receptor potential vanilloid type-1 (TRPV1) channels are expressed in vessels.5,6 TRPV1 channels are activated by the specific agonist, capsaicin, the ‘hot’ component of chili peppers.7,8 Activation of TRPV1 regulates the expression of endothelial cell-derived calcitonin gene-related peptide, which causes protective effects on vascular endothelial cells.8 We recently showed that chronic TRPV1 activation by dietary capsaicin increases the phosphorylation of protein kinase A (PKA) and endothelial nitric oxide (NO) synthase (eNOSser1177) and thus the production of NO in endothelial cells.9 Furthermore, our previous work indicated that activation of TRPV1 by capsaicin also affects lipid metabolism and prevents obesity in male mice.10 From these results, the hypothesis arises that activation of TRPV1 channels may attenuate atherosclerosis. We investigated this hypothesis in apolipoprotein E knockout mice (ApoE−/−) and ApoE/ TRPV1 double knockout mice (ApoE−/−/TRPV1−/−) on a high-fat diet. In vitro, activation of TRPV1 significantly reduced the accumulation of lipids in VSMC due to an increased cholesterol efflux and reduced cholesterol uptake. In vivo, long-term activation of TRPV1 significantly
2. Methods

Methods for cellular total cholesterol analysis, PCR, transient siRNA transfection, biochemical analyses, immunohistochemistry, evaluation of atherosclerotic lesions, and histological analysis are available in the Supplementary material online.

2.1 Genetic mouse models

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Experimental Animal Ethics Committee of Daping Hospital. ApoE-deficient mice (ApoE+/−) and TRPV1-deficient mice (TRPV1+/−) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The ApoE−/−TRPV1−/− mice were generated by breeding TRPV1−/− mice (on C57 BL/6 background) with ApoE−/− mice. The resulting heterozygous progeny were interbred with each other to produce homozygote ApoE−/−TRPV1−/− mice. PCR was used to identify the ApoE genotype and TRPV1 genotype. The expression level of TRPV1 protein was determined by western blot analysis.

To identify the ApoE genotype, the following set of primers was used: F: 5′-GCCTAGCGAGGGAGCGC-3′; R(WT): 5′-TGTAGCTTGGGA GCTCTGAGC-3′ and R(knock out): 5′-GCCGCCCCGACTGCAT CT-3′. For genotyping TRPV1, following three primers were used: F: 5′-CACAGACTGAGACAGCTT-3′; F(WT): 5′-CTTGTCACCAT GCTCTATT-3′; R(knock out): 5′-TCTCTAGCATGCTGAA AA-3′.

The male ApoE+/− and ApoE−/−TRPV1+/− mice were randomized into four groups: one group received standard laboratory chow, one group received a high-fat diet, one group received standard laboratory chow plus 0.01% capsaicin (Sigma-Aldrich), and one group received a high-fat diet plus 0.01% capsaicin for 24 weeks. The high-fat diet was supplemented with crude protein (18.8%), crude fat (16.2%), crude ash (5.2%), crude fibre (3.98%), nitrogen-free extract (45.2%), calcium (1.24%), phosphorus (0.83%), lysine (1.38%), and methionine/cystine (0.78%) (Shanghai Slac Laboratory Animal Co., Ltd, Shanghai, China). Food intake of all mice was measured. Mice were anaesthetized adequately by inhalation of isoflurane (5% for induction; 1% for maintenance) for blood collection and sacrificed by CO2 inhalation for isolation of the aorta. The adequacy of anaesthesia was monitored by testing tactile stimulus response and forelimb or hindlimb pedal withdrawal reflex, and continual observation of respiratory pattern, mucous membrane colour, and responsiveness to manipulations throughout all the procedure.

2.2 Cell culture

VSMC were obtained from thoracic aorta of mice and cultured by the tissue explant method as described.11 VSMC were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (HyClone) containing 100 µg/mL penicillin and 100 µg/mL streptomycin (GIBCO, USA). Cultured VSMC were plated and grew at 37°C in a humidified atmosphere of 95% air/5% CO2. To verify that cultured cells were VSMC, immunocytochemical localization of smooth muscle-specific α-actin was performed using anti-smooth muscle α-actin monoclonal antibody (Santa Cruz Biotechnology, USA). VSMC between Passages 2 and 6 were used. Quiescent VSMC were obtained by incubation with serum-free medium for 12 h before all the in vitro experimental procedures were performed.
Figure 1. Expression and function of TRPV1 on calcium influx in VSMC. (A) RT–PCR showing the expression of TRPV1 mRNA in the brain, aorta, and primarily cultured VSMC from C57BL/6J wild-type mice but not in those from TRPV1<sup>−/−</sup> mice and in a mouse macrophage cell line, RAW264.7. Predicted product size was 435 bp. (B) Immunoblotting showing TRPV1 protein expression in the brain, aorta, and VSMC from C57BL/6J wild-type mice, but not in those from TRPV1<sup>−/−</sup> mice and in RAW264.7. (C and D) Immunohistochemistry showing TRPV1 in the aorta (C) and VSMC (D) from C57BL/6J mice. Scale bar = 50 μm. (E) Effect of TRPV1 agonist capsaicin (Caps) on TRPV1 protein expression, VSMC were stimulated with 1 μmol/L Caps for 24 h and TRPV1 protein were analysed. Each n = 6; *P < 0.05. (F and G) Dose-dependent capsaicin induced calcium influx into VSMC from C57BL/6J mice. Representative fluorescence tracings (F) and summary of the data (G) are shown. Each n = 4–6; *P < 0.05; **P < 0.01. (H and I) Caps (1 μmol/L) induced calcium influx into VSMC from C57BL/6J mice (Control), but not in VSMC from TRPV1<sup>−/−</sup> mice or after pre-treatment with capsazepine (1 μmol/L, Capz) or 5′-iodo-RTX (1 μmol/L) for 10 min. Representative fluorescence tracings (H) and summary of the data (I) are shown. Each n = 4–6; *P < 0.05; **P < 0.01.
the presence and functional integrity of TRPV1 channels in VSMC, extending the observations in recent literature. 13

3.2 Activation of TRPV1 by capsaicin reduces accumulation of lipids in VSMC

There is indirect evidence linking TRPV1 with lipid storage and lipid metabolism.10,14 Therefore, we investigated the effects of TRPV1 activation on lipid accumulation in VSMC. Oil red O staining of intracellular lipid droplets showed that the administration of 50 μg/mL oxidized LDL (oxLDL) for 72 h significantly increased lipid accumulation in cultured VSMC from C57BL/6J mice by about 154 ± 13% from n = 6 separate experiments (Figure 2A). Scale bar = 50 μm. (B and D) Bar graphs showing total cholesterol levels in VSMC from C57BL/6J and TRPV1−/− mice under routine culturing conditions (C) and after culturing in the presence of 50 μg/mL oxLDL for 3 days (D). Total intracellular cholesterol was measured in VSMC from C57BL/6J and TRPV1−/− mice cultured in the absence (Control) or presence of Caps or Capz. Data are mean ± SEM from five to six independent experiments. *P < 0.05 compared with Control.

3.3 Activation of TRPV1 affects cholesterol transporters in VSMC

After we identified the regulatory effect of TRPV1 on total cellular cholesterol level, we investigated whether TRPV1 activation may directly affect cholesterol transporters that are associated with intracellular cholesterol accumulation. First, we showed that both ABCA1 and LRP1 are colocalized with TRPV1 on VSMC (Figure 3A and B). Furthermore, oil red O staining of intracellular lipid droplets showed that RNA interference knockdown of ABCA1 reversed capsaicin-induced reduction in lipid accumulation in cultured VSMC from C57BL/6J mice (see Supplementary material online, Figure S2A), and RNA interference knockdown of LRP1 had the synergistic effect with capsaicin in attenuated lipid accumulation in VSMC (see Supplementary material online, Figure S2B).
The activation of TRPV1 by the specific agonist capsaicin significantly increased the expression of ABCA1 from 1.00 + 0.06 to 2.18 + 0.20 \((n = 3, P < 0.05; \text{Figure 3C})\), whereas it significantly reduced the expression of LRP1 from 1.00 + 0.20 to 0.53 + 0.08 \((n = 6, P < 0.05; \text{Figure 3D})\). However, these effects of capsaicin disappeared in VSMC from TRPV1\(^{-/-}\) mice (Figure 3C and D).

### 3.4 TRPV1 activation regulates ABCA1/LRP1 expression by calcium-evoked calcineurin- and PKA-dependent mechanisms in VSMC

For further understanding of the possible molecular mechanisms of ABCA1 and LRP1 expression regulated by TRPV1 activation, we detected the role of PKA and calcineurin in the expression of ABCA1 and LRP1 stimulated by capsaicin. Cyclosporin A (CsA), a specific inhibitor of calcineurin, had an inhibitory tendency on capsaicin-activated ABCA1 expression (Figure 4A), and blocked the effects of TRPV1 activation on LRP1 expression \((0.57 + 0.06 \text{ vs.} 1.02 + 0.08; n = 3, P < 0.05; \text{Figure 4B})\). As shown in Supplementary material online, Figure S4, capsaicin increased the expression of phospho-PKA. KT5720, a specific inhibitor of PKA, blocked the effects of TRPV1 activation on ABCA1 \((1.35 + 0.06 \text{ vs.} 0.83 + 0.07; n = 3, P < 0.01; \text{Figure 4C})\) and LRP1 expression \((0.34 + 0.05 \text{ vs.} 0.71 + 0.10; n = 3, P < 0.05; \text{Figure 4C})\). As expected, trapping of intracellular calcium by BAPTA blocked the effects of capsaicin on ABCA1 and LRP1 expression (Figure 4D). However, peroxisome proliferator activated receptor gamma (PPAR\(\gamma\)) antagonist, GW9662, did not affect ABCA1 expression after activation of TRPV1 (see Supplementary material online, Figure S5). These results in vitro indicated that TRPV1 activation increased ABCA1 expression and reduced LRP1 expression, leading to increased cholesterol efflux and reduced cholesterol uptake of VSMC, through calcium-dependent calcineurin signal and PKA phosphorylation mechanisms.
3.5 In vivo activation of TRPV1 increases ABCA1 expression and reduces LRP1 expression in aorta from ApoE<sup>−/−</sup> mice

To confirm these effects in vivo, we evaluated the effects of TRPV1 activation on cholesterol transporters in ApoE<sup>−/−</sup> mice. Table 1 showed the characteristics of ApoE<sup>−/−</sup> mice that were randomly allocated to a normal diet, a high-fat diet, normal diet plus capsaicin, or a high-fat diet plus capsaicin, respectively. Compared with mice on normal diet, administration of a high-fat diet significantly increased plasma triglycerides by 300% and total cholesterol by 72% in ApoE<sup>−/−</sup> mice (<i>P</i> < 0.01; Table 1). Compared with mice on a high-fat diet, the mice on a high-fat diet plus capsaicin showed significantly lower plasma triglycerides (2.39 ± 0.50 vs. 4.17 ± 0.74 mmol/L, <i>P</i> < 0.05 Table 1) and significantly lower total cholesterol (13.75 ± 2.17 vs. 18.62 ± 1.54 mmol/L, <i>P</i> < 0.05; Table 1). In ApoE<sup>−/−</sup> mice on a high-fat diet, capsaicin did not affect fasting plasma glucose or insulin (Table 1). No difference in plasma lipids was shown in ApoE<sup>−/−</sup> TRPV1<sup>−/−</sup> mice fed with different diets (see Supplementary material online, Figure S6). Using immunofluorescence, we confirmed the expression of TRPV1, ABCA1, and LRP1 in the aorta from ApoE<sup>−/−</sup> mice (Figure 5A and B). Compared with ApoE<sup>−/−</sup> mice on a high-fat diet, the ApoE<sup>−/−</sup> mice on a high-fat diet plus capsaicin showed significantly higher expression of ABCA1 in the aorta (5.60 ± 1.62 vs. 1.32 ± 1.21, <i>n</i> = 3, <i>P</i> < 0.05; Figure 5C) and reduced expression of LRP1 in the aorta (0.55 ± 0.04 vs. 0.81 ± 0.04; <i>n</i> = 3, <i>P</i> < 0.05; Figure 5D), and similar changes of ABCA1 and LRP1 in the aorta between ApoE<sup>−/−</sup> mice on normal diet and...
Table 1  Biochemical characteristics of ApoE<sup>−/−</sup> mice after 24 weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>ND (n = 20)</th>
<th>NCaps (n = 15)</th>
<th>HD (n = 16)</th>
<th>HCaps (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>10.81 ± 0.58</td>
<td>7.57 ± 0.56</td>
<td>18.62 ± 1.54**</td>
<td>13.75 ± 2.17&amp;&amp;</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.04 ± 0.08</td>
<td>0.66 ± 0.06</td>
<td>4.17 ± 0.74**</td>
<td>2.39 ± 0.50&amp;##</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.69 ± 0.31</td>
<td>1.53 ± 0.02</td>
<td>2.19 ± 0.40</td>
<td>2.56 ± 0.38</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>1.72 ± 0.22</td>
<td>1.11 ± 0.06</td>
<td>2.22 ± 0.37</td>
<td>2.15 ± 0.20&amp;</td>
</tr>
<tr>
<td>Insulin (IU/L)</td>
<td>13.24 ± 1.99</td>
<td>10.62 ± 1.33</td>
<td>12.14 ± 0.96</td>
<td>13.82 ± 2.64</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.41 ± 0.74</td>
<td>7.45 ± 0.77</td>
<td>6.41 ± 0.58</td>
<td>7.51 ± 0.83</td>
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</table>

ND, normal diet; NCaps, normal diet plus capsaicin; HD, high-fat diet; HCaps, high-fat diet plus capsaicin; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol. Values are expressed as mean ± SEM for 15–20 animals.

**P < 0.01 vs. ND group.
#P < 0.05 vs. HD group.
&##P < 0.05 vs. NCaps group.

Figure 5  In vivo activation of TRPV1 affects cholesterol transporters in the aorta. ApoE<sup>−/−</sup> mice were randomly allocated to four groups, which were fed for 24 weeks with normal diet (ND), normal diet plus capsaicin (NCaps), high-fat diet (HD), or high-fat diet plus capsaicin (HCaps), respectively. (A and B) Immunofluorescence showing the colocalization of ABCA1 (A) and LRP1 (B) with TRPV1 on the surface of the aorta from C57BL/6J mice. ABCA1, LRP1, and TRPV1 were identified using specific primary antibodies and fluorescence-labelled secondary antibodies. Negative controls (Control) were performed with PBS instead of primary antibodies. Scale bar = 200 μm. (C and D) Representative immunoblottings and summary data showing the in vivo effect of capsaicin on the expression of ABCA1 (C) and LRP1 (D) in the aorta from ApoE<sup>−/−</sup> mice. Data are mean ± SEM of aortas from ApoE<sup>−/−</sup> mice randomly allocated to ND, NCaps, HD, or HCaps for 24 weeks, respectively. Each n = 3; *P < 0.05 by ANOVA.
ApoE<sup>−/−</sup> mice on normal diet plus capsaicin were also observed (Figure 5C and D). On the other hand, long-term administration of capsaicin did not affect the expressions of other cholesterol transporters and receptors, such as scavenger receptor type A (SR-A), ATP-binding cassette subfamily G member 1 (ABCG1), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and caveolin-1 (Cav-1) in ApoE<sup>−/−</sup> mice (see Supplementary material online, Figures S7 and S8).

### 3.6 TRPV1 reduces lipid storage and atherosclerotic lesions in ApoE<sup>−/−</sup> but not ApoE<sup>−/−</sup> TRPV1<sup>−/−</sup> mice on a high-fat diet

To prove that TRPV1 directly affects atherosclerosis, we compared the effects of chronic activation of TRPV1 on atherosclerotic lesions from ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> TRPV1<sup>−/−</sup> mice. PCR was used to identify the ApoE genotype (Figure 6A) and TRPV1 genotype (Figure 6B). Food intake showed differences between mice fed with and without capsaicin only during the first 4 days, then mice fed with capsaicin recovered to normal eating (Figure 6C and D). As shown in Supplementary material online, Figure S9A and Figure 6E, lipid storage in atherosclerotic lesions of the descending thoracoabdominal aorta of ApoE<sup>−/−</sup> mice and ApoE<sup>−/−</sup> TRPV1<sup>−/−</sup> mice. Each n = 6; **P < 0.01. (F) Bar graphs showing atherosclerotic lesions in aortic sinus of consecutive sections of the haematoxylin–eosin-stained aortic root quantified by computer-assisted image analysis. Data are mean ± SEM. Each n = 5–10; **P < 0.01.
channels have also been demonstrated in vascular system, including endothelial cells and VSMC. Currently, it is unknown whether TRPV1 channels are uniformly distributed in all cell types from different blood vessels, including the aorta, coronary arteries, or mesenteric arteries. Chronic activation of TRPV1 improves endothelium-dependent vasodilatation through increasing PKA and eNOS phosphorylation. Tissue-specific activation of TRPV1 channels may mediate endothelium-dependent vasodilatation or smooth-muscle-associated vasoconstriction. In the present study, we not only confirmed the expression of TRPV1 mRNA and protein in VSMC and aorta, but also confirmed the expected molecular mass of TRPV1 of 95 kDa and showed that the antibodies were able to identify TRPV1 by immunoblots.

The capsaicin-induced calcium influx through TRPV1 channels has already been reported in HEK293 cells transfected with encoding cDNA of TRPV1. Moreover, capsaicin, as a known specific agonist of TRPV1 channels, induced a dose-dependent calcium influx into VSMC. As reported, TRPV1 activity is involved in calcineurin pathway. A similar mechanism of calcineurin—nuclear factor-activated T cells (NFAT)-dependent activation of TRPC3 and TRPC6 gene expression has also recently reported in myocardial cells. Similar to those reports, we showed that specific activation of TRPV1 by capsaicin could not only cause transmembrane calcium influx and increase the expression of TRPV1, but also reduce the accumulation of lipids in VSMC. Which mechanisms mediate the reduced intracellular lipid accumulation after TRPV1 activation? Previous reports showed that elevated cytosolic calcium markedly suppressed intracellular lipid accumulation and cholesterol and triglyceride levels in adipocytes. The capsaicin-induced calcium influx through increasing ABCA1 expression and decreasing LR1P expression in cultured VSMC, as a result, reduced intracellular lipid droplets and cholesterol levels in VSMC, as well as in the aorta from ApoE−/− mice on a high-fat diet. However, the regulation effects of capsaicin were not observed on other cholesterol transporters and receptors, such as SR-A, ABCG1, LOX-1, and Cav-1 in the aorta, although they were also considered playing roles in intracellular lipid homeostasis in the procedure of atherogenesis. In recent literature, cholesterol-lowering intervention by simvastatin downregulated the overexpression of vascular LR1P induced by hypercholesterolaemia and that simvastatin did not influence LR1P expression beyond its cholesterol-lowering effects in male New Zealand rabbits. According to our present data, in vivo in mice and in cultured cells, the observed effects of capsaicin are primarily caused by its activation of TRPV1 channels, rather than indirectly by lowering hypercholesterolaemia, although we observed lower plasma triglycerides and total cholesterol after administration of capsaicin in vivo. It cannot be excluded that several TRPV1-associated mechanisms collaborate in vivo. In vivo, both the TRPV1-associated reduction in plasma cholesterol including the reduction in remnant lipoprotein particles and the TRPV1-associated increase in ABCA1 expression and reduction in LR1P expression in VSMC will result in prevention of atherosclerosis.

The underlying mechanisms of calcium-dependent regulation of cholesterol transporter are supported by previous studies. PPARγ enhances cholesterol efflux by inducing the transcription of liver-X-receptor α and thus inducing ABCA1 expression. Inhibiting calcineurin by specific immunosuppressant sirolimus with CsA was reported to downregulate ABCA1 protein expressions. Kiss et al. and Zhu and Hui showed that ABCA1 and LR1P were
regulated by PKA, which is calcium-dependent. Taken together, we supposed that increased cytosolic calcium will modulate PPARγ-, calcineurin-, and PKA-dependent pathways, thus will finally change cholesterol transporters expression. However, our experimental data using PPARγ antagonist showed that the increased ABCA1 expression after activation of TRPV1 may not be explained by PPARγ mechanism. Moreover, our research elucidated that TRPV1 activation increased ABCA1 expression and reduced LRPI expression through calcineurin- and PKA-dependent mechanisms, which were calcium-evoked, thus leading to increased cholesterol efflux and reduced cholesterol uptake into VSMC. The capsaicin content was found to range from 2.19 to 19.73 mg/g of dry weight of capiscum fruits. Moreover, one human study showed that regular consumption of chili (30 g/day; 55% cayenne chili) for 4 weeks may attenuate postprandial hyperinsulinemia. Based on these values, one may estimate that average 18 g dried chili (about 180 mg capsaicin) per day would be beneficial to humans.

In summary, our present study for the first time gives experimental evidence that continuous activation of TRPV1 seems to be a promising novel mechanism to attenuate atherosclerosis evoked by a high-fat diet.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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