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RESEARCH PAPER

Eugenol dilates mesenteric arteries and reduces systemic BP by activating endothelial cell TRPV4 channels

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BACKGROUND AND PURPOSE

Eugenol, a vanilloid molecule found in some dietary plants, relaxes vasculature in part via an endothelium-dependent process; however, the mechanisms involved are unclear. Here, we investigated the endothelial cell-mediated mechanism by which eugenol modulates rat mesenteric artery contractility and systemic BP.

EXPERIMENTAL APPROACH

The isometric tension of rat mesenteric arteries (size 200–300 μm) was measured using wire myography; non-selective cation currents (I_{Cat}) were recorded in endothelial cells using patch clamp electrophysiology. Mean arterial pressure (MAP) and heart rate (HR) were determined in anaesthetized rats.

KEY RESULTS

Eugenol relaxed endothelium-intact arteries in a concentration-dependent manner and this effect was attenuated by endothelium denudation. L-NAME, a NOS inhibitor, a combination of TRAM-34 and apamin, selective blockers of intermediate and small conductance Ca^{2+}-activated K^+ channels, respectively, and HC-067047, a TRPV4 channel inhibitor, but not indomethacin, a COX inhibitor, reduced eugenol-induced relaxation in endothelium-intact arteries. Eugenol activated HC-067047-sensitive I_{Cat} in mesenteric artery endothelial cells. Short interfering RNA (siRNA)-mediated TRPV4 knockdown abolished eugenol-induced I_{Cat} activation. An i.v. injection of eugenol caused an immediate, transient reduction in both MAP and HR, which was followed by prolonged, sustained hypotension in anaesthetized rats. This sustained hypotension was blocked by HC-067047.

CONCLUSIONS AND IMPLICATIONS

Eugenol activates TRPV4 channels in mesenteric artery endothelial cells, leading to vasorelaxation, and reduces systemic BP *in vivo*. Eugenol may be therapeutically useful as an antihypertensive agent and is a viable molecular candidate from which to develop second-generation TRPV4 channel activators that reduce BP.

Abbreviations

EDHF, endothelium-derived hyperpolarizing factor; I_{Cat}, non-selective cation currents; IK_{Ca}, intermediate conductance calcium-activated potassium channel; MAP, mean arterial pressure; PGI_2, prostacyclin; siRNA, short interfering RNA; SK_{Ca}, small conductance calcium-activated potassium channel; TRP, transient receptor potential

Tables of Links

TARGETS	
GPCRs[a]	**Ion channels**[b]
TXA_2 (TP) receptor	BK (K_{Ca} 1.1) channel
Enzymes[c]	IK_{Ca} (K_{Ca} 3.1) channel
COX	SK_{Ca} (K_{Ca} 2.1) channel
NOS	TMEM16A (CaCC)
	TRPV4

LIGANDS		
ACh	GSK1016790A	Phenylephrine
Apamin	HC-067047	PGI_2
Capsaicin	Indomethacin	TRAM-34
Eugenol	L-NAME	U46619
	Nitric oxide (NO)	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Endothelial cells line the luminal surface of all blood vessels and regulate vascular tone, coagulation and fibrinolysis, vascular inflammation and angiogenesis (Félétou and Vanhoutte, 2009). The endothelium regulates vascular tone by releasing factors, including NO, in response to both mechanical forces and soluble agonists. Endothelial intracellular calcium (Ca^{2+}) concentration regulates vascular tone by controlling the generation of NO produced by NOS, prostacyclin (PGI_2) generated by COX, and endothelium-derived hyperpolarizing factor (EDHF) (Favero *et al.*, 2014). Endothelial cells also regulate vascular tone by controlling the membrane potential of electrically coupled smooth muscle cells (Yamamoto *et al.*, 1999; Sandow and Hill, 2000; Haddock *et al.*, 2006).

Several members of the transient receptor potential (TRP) family of non-selective cation channels are expressed in endothelial cells and are involved in endothelium-dependent vasodilatation (Garcia and Schilling, 1997; Earley and Brayden, 2010; Zhang and Gutterman, 2011). TRPV4, a vanilloid (TRPV) family member, is a Ca^{2+}-permeable ion channel expressed in endothelial cells that stimulates vasodilator mechanisms (Hartmannsgruber *et al.*, 2007; Saliez *et al.*, 2008; Mendoza *et al.*, 2010; Sonkusare *et al.*, 2012). TRPV4 channels are activated by a broad range of chemical and physical stimuli (Filosa *et al.*, 2013). Several studies have demonstrated that TRPV4 channels control vascular tone and influence BP (Watanabe *et al.*, 2002; Kohler *et al.*, 2006; Earley *et al.*, 2009; Zhang *et al.*, 2009; Ma *et al.*, 2013).

Eugenol is a natural compound found in dietary plants including cloves, basil, cinnamon and nutmeg (Kamatou *et al.*, 2012). These plants possess antihypertensive properties, although the chemicals mediating this effect have not been elucidated (Grover *et al.*, 2002; Umar *et al.*, 2010; Ranasinghe *et al.*, 2013). Eugenol and capsaicin share a vanillyl group as an important structural motif for bioactivity. Therefore, eugenol, like capsaicin, can stimulate TRPV channels (Calixto *et al.*, 2005). Eugenol activates TRPV1 channels in trigeminal ganglion neurons and TRPV3 channels in keratinocytes and endothelial cells (Yang *et al.*, 2003; Xu *et al.*, 2006; Earley and Brayden, 2010; Earley *et al.*, 2010). In normotensive and deoxycorticosterone acetate-salt hypertensive rats, eugenol reduces BP and induces bradycardia. Both studies suggested that eugenol-induced hypotension may occur because of vasorelaxation (Lahlou *et al.*, 2004; Interaminense *et al.*, 2007). Eugenol also relaxed rat aorta and an intact mesenteric bed. The effects were shown to be partially dependent on the endothelium, but their mechanisms of action were unclear (Criddle *et al.*, 2003; Damiani *et al.*, 2003).

Here, we investigated endothelial-dependent eugenol-induced vasodilatation in rat mesenteric arteries, the mechanisms involved and functional significance. Our data indicate that eugenol activates TRPV4 currents in mesenteric artery endothelial cells, leading to vasodilatation and a reduction in systemic BP.

Methods

Tissue preparation

Animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center (Memphis, TN, USA) and Universidade Estadual do Ceará (Fortaleza, CE, Brazil). In this study, the total of 40 male Wistar rats (250 g) were used. Animals were killed by an i.p. injection of sodium pentobarbital solution (150 $mg \cdot kg^{-1}$). The mesenteric bed was removed and third-order branches (200–300 µm diameter) were harvested in ice-cold Krebs–Hanseleit solution (KHS) of composition (in $mmol \cdot l^{-1}$): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2.2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4.7H_2O$ 1.2; EDTA 0.01; glucose 11. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Vascular reactivity experiments

Mesenteric artery segments (2 mm in length) were mounted in a small vessel myograph chamber (model 610M; Danish Myo Tech, Aarhus, Denmark) for isometric tension recordings. Briefly, two steel wires (40 µm diameter) were introduced through the lumen and mounted according to methods previously described (Mulvany and Halpern, 1977). After a 15 min equilibration period in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal

lumen diameter for active tension development. This was determined based on the internal circumference/wall tension ratio of the segments by setting the internal circumference, L_1, to 90% of a value determined by a passive tension equivalent to a transmural pressure of 100 mmHg (Mulvany and Halpern, 1977). The diameter (I_1) was determined according to the equation $I_1 = L_1/\pi$, using specific software for normalization of resistance arteries (DMT Normalization Module; AD Instruments, Sydney, Australia). Segments were left to equilibrate for 30 min. Vessel contractility was then tested by an initial exposure to a high-K^+ (120 mmol·l^{-1}) solution. Where required, endothelium was denuded by introducing an air bubble into the artery lumen for 1 min followed by a wash with KHS. Endothelium removal was confirmed by the absence of relaxation to ACh (10 μM) in phenylephrine (PE, 1–10 μM) precontracted arteries.

Arteries were precontracted using U46619 (1 μM) to produce a maximal, sustained contraction similar to that stimulated by KCl (120 mmol·l^{-1}) (Rossoni *et al.*, 2011). After the contraction stabilized, increasing cumulative concentrations of eugenol (1–1000 μM) were applied. Effects of eugenol (100 μM) were also studied on U46619 (1 μM) precontracted arteries in the presence of L-NAME (100 μM), a NOS inhibitor; indomethacin (10 μM), a COX inhibitor; a combination of TRAM-34 (1 μM) and apamin (1 μM), intermediate and small conductance Ca^{2+}-activated K^+ channel inhibitors, respectively, or HC-067047 (100 nM), a TRPV4 inhibitor.

Cell culture and TRPV4 knockdown

Rat mesenteric artery endothelial cells (Cell Biologics, Chicago, IL, USA) of passage 2 were maintained as recommended by the supplier under standard tissue culture condition (21% O_2–5% CO_2; 37°C). Short interfering RNAs (siRNA; Silencer® Select siRNA, Ambion®, Life Technologies, Carlsbad, CA, USA) specifically targeting TRPV4 were inserted into endothelial cells by transient transfection using Effectene® reagent (Qiagen, Valencia, CA, USA). Sense and anti-sense nucleotide sequences for TRPV4 siRNA were: AGAGAACCCUCACAAGAAAtt and UUUCUUGUGAGGGUUCUCUgt respectively. All experimental measurements were performed 36–48 h post-transfection. Non-targeting siRNA (Silencer® Select Negative Control n° 1, Ambion®, Life Technologies) was used as a negative control.

Western blotting

Cultured endothelial cell lysate protein concentration was determined spectrophotometrically with amido black solution. Proteins (<40 μg per lane) were separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Blots were physically cut at 75 kDa to permit probing for TRPV4 at a higher MW and actin at the lower MW. Membranes were incubated with rabbit polyclonal anti-TRPV4 (1:1000, Abcam, Cambrigde, UK) or anti-actin (1:5000, EMD Millipore, Billerica, MA, USA) primary antibodies overnight at 4°C in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk. Proteins were visualized using HRP-conjugated secondary antibody (1:10,000 dilution; Pierce, Rockford, IL, USA) and a chemiluminescent detection kit (Pierce, Rockford, IL, USA).

Patch clamp electrophysiology

Non-selective cation currents (I_{Cat}) were recorded in cultured endothelial cells of rat mesenteric artery. Endothelial cells were trypsinized and allowed to adhere to glass coverslips for 2 h at 37°C. Coverslips were transferred to a recording chamber and whole-cell patch clamp recordings were acquired at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and pCLAMP 8.2 (Molecular Devices, Sunnyvale, CA, USA). Borosilicate glass electrodes of resistance 3–6 MΩ were filled with pipette solution containing (in mmol·L^{-1}): 120 NaGlutamate, 20 NaCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, 4 Na_2ATP (pH 7.2). The extracellular bath solution contained (in mmol·L^{-1}): 142 NaCl, 2 $CaCl_2$, 6 KCl, 1 $MgCl_2$, 10 HEPES and 10 glucose. Isolated cells with intact membranes that were not attached to neighbouring cells were used for patch clamp electrophysiology. Cell capacitance was measured by application of a 5 mV test pulse and correcting transients with series resistance compensation. I_{Cat} were recorded by applying voltage ramps (940 ms) every 20 s, ramping between −120 and +100 mV, from a 0 mV holding potential. Whole-cell currents were filtered at 1 kHz and digitized at 5 kHz.

In vivo *BP measurement*

Male Sprague-Dawley rats (250 g) were anaesthetized by an initial i.p. injection of sodium pentobarbital (50 mg·kg^{-1}). Anaesthesia was maintained through i.v. injection of sodium pentobarbital based on constant monitoring of reflexes in response to hindlimb pinching or blinking evoked by a low-pressure corneal stimulation. Catheters (PE-10 fused to PE-50) filled with heparin (125 IU·mL^{-1})-treated saline solution were implanted in the abdominal aorta to record arterial BP and in the inferior vena cava for drug administration, through the left femoral artery and vein respectively. The aortic catheter was connected to a BP transducer (Living System Instrumentation, Burlington, VT, USA) coupled to an interface and software (Power Lab/8SP; AD Instruments) for systolic and diastolic pressure acquisition at a sampling frequency of 1 kHz. Mean arterial pressure (MAP) was calculated as diastolic pressure + [(systolic – diastolic)/3]. Heart rate was determined from pressure pulse intervals. Animals were allowed to stabilize for 15 min before starting experiments. Baseline values of MAP and heart rate were determined and changes measured during a 30 min post-injection period. Eugenol (5 mg·kg^{-1}, i.v.) was administered as a bolus (100 μL) followed by a 50 μL flush with physiological saline. The effects of eugenol on BP took ~45 min to reverse. Ten min after BP returned to baseline, HC-067047 (9 μg·kg^{-1}, i.v.) was injected. Eugenol was then applied i.v. 5 min after HC-067047.

Chemicals

PE, ACh and L-NAME were first diluted in distilled water to 10^{-1} M. U46619 and HC-067047 were diluted in ethanol to 10^{-2} M. Indomethacin and apamin were first dissolved in acetic acid (0.05 M) and tris-HCl solution (pH 8.0), respectively, to 10^{-2} M. TRAM-34 was diluted in DMSO to 10^{-2} M. For *in vitro* experiments, eugenol (Sigma code #E51791) was first diluted in DMSO, brought to volume with KHS and sonicated immediately before use. For *in vivo* experiments, eugenol was dissolved in Tween 80 (2%), brought to final volume (100 μL) with sterile isotonic saline and sonicated before use. Final

concentrations of ethanol and DMSO were ≥0.2%. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). HC-067047 and TRAM-34 were purchased from Tocris Bioscience (Bristol, UK).

The drug/molecular target nomenclature conforms to BJP's Concise Guide to PHARMACOLOGY (Alexander *et al.*, 2013a,b,c).

Statistical analysis

Data are expressed as means ± SEM. Individual eugenol concentration–response curves and mean concentration–response data were both fit with non-linear regression analysis using a four parameter logistic equation to calculate EC_{50} and Hill slope. Statistical significance was calculated by using Student's *t*-tests for paired or unpaired data and ANOVA followed by Bonferroni's *post hoc* test for multiple datasets. $P < 0.05$ was considered significant.

Results

Eugenol induces endothelium-dependent relaxation of rat mesenteric arteries

Mesenteric artery rings with intact endothelium were mounted and a contraction was induced by U46619, a thromboxane A_2 receptor agonist, with a mean tension development of 3.8 ± 0.2 $mN \cdot mm^{-1}$ (Figure 1A). Bath application of eugenol caused concentration-dependent relaxation of this contraction, which was significant at concentrations higher than 30 μM (Figure 1C). Non-linear regression analysis of individual concentration-response experiments produced a mean EC_{50} of 47.9 ± 6.3 μM and a slope of 4.0 ± 0.6. The vehicle (DMSO) for eugenol did not alter arterial contractility. The highest DMSO concentration used, which was that needed to dissolve 1 mM eugenol, alone did not alter contractility (94.8 ± 5.7% of control force, $n = 8$, $P > 0.05$).

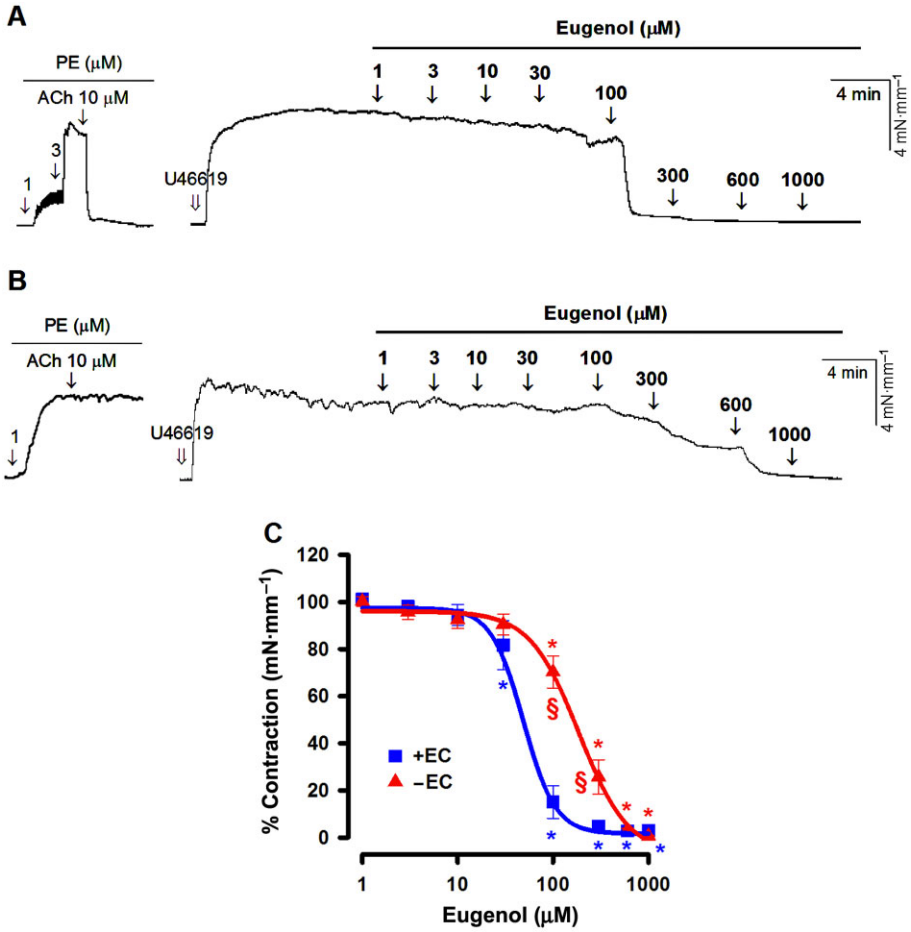

Figure 1

Eugenol relaxes endothelium-intact and -denuded mesenteric arteries. (A) Representative recording of concentration-dependent eugenol-induced relaxation in an endothelium-intact artery precontracted with U46619 (1 μM). The left trace shows the ACh (10 μM) -induced relaxation of a phenylephrine (PE)-induced contraction in the same artery. (B) Representative recording of concentration-dependent eugenol-induced relaxation in an endothelium-denuded artery precontracted by U46619 (1 μM). The left trace illustrates the lack of an ACh (10 μM)-induced relaxation of a PE-induced contraction in the same artery. (C) Mean data fit with non-linear regression analysis: endothelium-intact (+EC, $n = 9$) and -denuded (-EC, $n = 5$). *,§$P < 0.05$ versus control and +EC respectively.

Endothelium denudation significantly attenuated eugenol-induced vasorelaxation (Figure 1B,C). In endothelium-denuded arteries, eugenol-induced relaxation was significant at concentrations higher than 100 μM, with an EC_{50} of 189.9 ± 19.3 μM and slope of 2.1 ± 0.4 (Figure 1B,C). These data indicate that eugenol relaxes mesenteric arteries, in part, via an endothelium-mediated mechanism.

Endothelium-dependent eugenol-induced relaxation involves NOS, K_{Ca} and TRPV4 channels

To investigate the endothelial cell-mediated mechanism that contributes to eugenol-induced relaxation, experiments were performed in the presence L-NAME, a NOS inhibitor, indomethacin, a COX inhibitor, TRAM-34 and apamin, inhibitors of intermediate (IK_{Ca}) and small conductance (SK_{Ca}) Ca^{2+}-activated K^+ channels, respectively, or HC-067047, a TRPV4 blocker. Relaxation to eugenol was measured in both the absence and presence of these inhibitors in the same endothelium-intact mesenteric arteries.

Each blocker caused a small relaxation, except L-NAME which increased tone (Supporting Information Fig. S1). L-NAME, a combination of TRAM-34 and apamin, or HC-067047 each attenuated eugenol-induced vasorelaxation to ~55.5, 58.2 and 46.4%, respectively, without altering the relaxation rate (Figure 2A,B, Supporting Information Table S1). Eugenol-induced relaxation in HC-067047 + L-NAME or HC-067047 + TRAM/apamin was similar to that in the presence of each blocker alone, indicating a similar mechanism is involved (Figure 2B). In contrast, indomethacin did not alter the eugenol-induced relaxation (Figure 2A,B). Repetitive applications of eugenol caused similar-sized relaxation responses (first application, 55.7 ± 6.5% relaxation; second application, 62.2 ± 7.6% relaxation; $n = 6$, $P > 0.05$), indicating that the altered responses in the presence of the blockers was not due to desensitization of the eugenol effect (Figure 2D). To investigate whether eugenol-induced, endothelial-independent relaxation occurred through TRPV4, SK_{Ca} and IK_{Ca} channels, similar experiments were performed in endothelium-denuded arteries. HC-067047 reduced eugenol-induced relaxation in endothelium-denuded arteries to ~73.6% of control (Figure 2C). In contrast, TRAM-34/apamin did not alter eugenol-induced relaxation in endothelium-denuded arteries

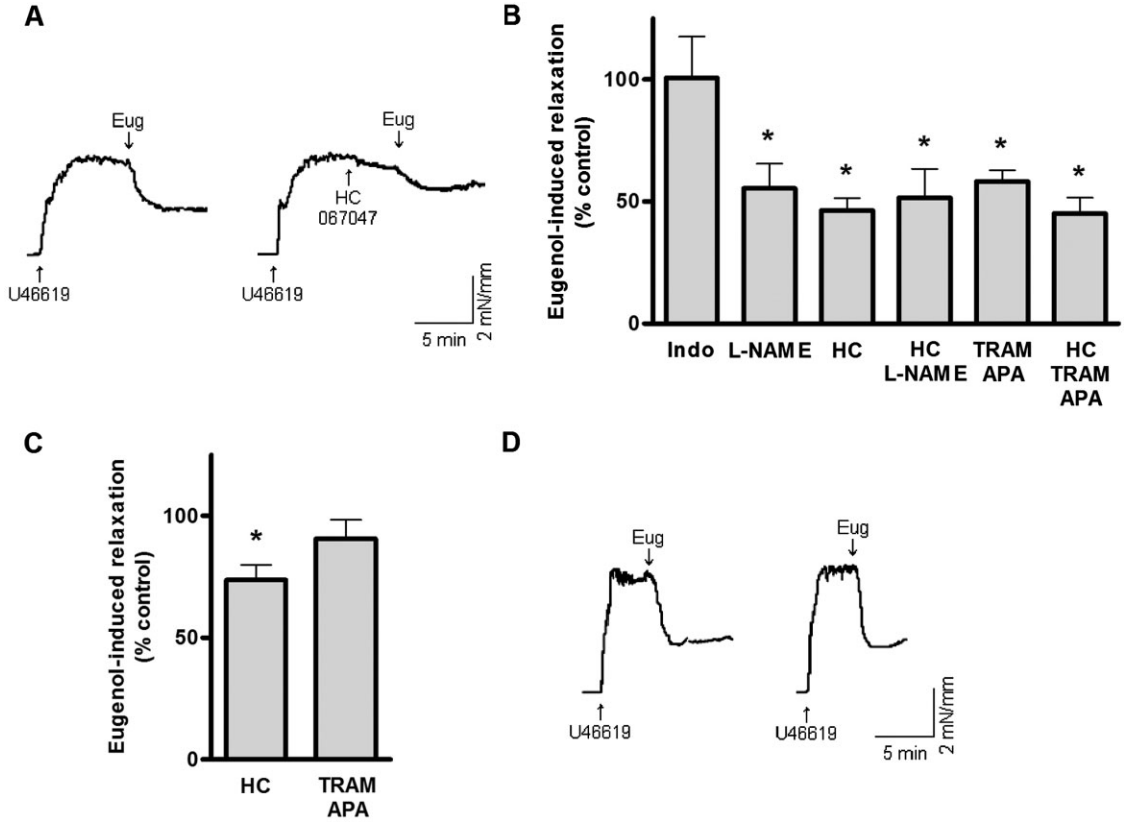

Figure 2

Inhibitors of endothelial signalling attenuate eugenol-induced vasorelaxation. (A) Representative recordings of eugenol (100 μM)-induced relaxation alone or in the presence of HC-067047 (100 nM) in the same endothelium-intact artery. (B) Mean data for the dilatation induced by eugenol (100 μM) in endothelium-intact arteries in the presence of: indomethacin (10 μM, $n = 6$), L-NAME (100 μM, $n = 5$), HC-067047 (100 nM, $n = 6$), HC-067047 plus L-NAME ($n = 5$), TRAM-34 plus apamin (1 μM each, $n = 6$), or HC-067047 plus TRAM-34/apamin ($n = 6$). *$P < 0.05$ versus eugenol alone. (C) Mean data for eugenol (100 μM)-induced dilatation in endothelium-denuded arteries precontracted by U46619 (1 μM) in the presence of HC-067047 (100 nM, $n = 7$) or TRAM-34 plus apamin (1 μM each, $n = 6$). *$P < 0.05$ versus eugenol alone. (D) Representative recording of two eugenol (100 μM)-induced relaxations in the same endothelium-intact artery.

(Figure 2C). These data indicate that endothelial NOS, SK_{Ca} and IK_{Ca} channels and TRPV4, but not COX, are involved in eugenol-induced endothelium-dependent vasorelaxation. Eugenol-induced TRPV4 channel activation also contributes to endothelium-independent relaxation.

Eugenol stimulates TRPV4-mediated currents in mesenteric artery endothelial cells

We focused on directly testing the hypothesis that eugenol activates TRPV4 channels by performing patch clamp electrophysiological experiments on mesenteric artery endothelial cells. I_{Cat} were stimulated by applying repetitive 940 ms ramp pulses from −120 to +100 mV from a holding potential of 0 mV. Eugenol increased mean I_{Cat} (at −100 mV) from −14.7 ± 2.17 pA to −21.8 ± 4.02 pA or 1.48-fold (Figure 3A,B). HC-067047, a TRPV4 inhibitor, alone did not alter I_{Cat}, but blocked eugenol activation of I_{Cat} (Figure 3A,B).

Eugenol-induced I_{Cat} activation was also studied in endothelial cells in which TRPV4 expression was reduced using siRNA, with control siRNA used as a control. TRPV4 siRNA reduced TRPV4 protein to ~52% of control, but did not alter basal current density (pA pF^{-1}: control 1.5 ± 0.3, TRPV4 siRNA, 1.8 ± 0.1, n = 5 for each, Figure 3C,D). GSK1016790A (10 nM), a TRPV4 agonist, increased mean I_{Cat} ~3.54-fold in TRPV4 siRNA-treated cells, but only 1.22-fold in control cells (Figure 3E). These data indicate that endothelial cells were functional after transfection and TRPV4 siRNA reduced TRPV4 expression and currents. Eugenol increased mean I_{Cat} ~1.45-fold in cells transfected with control siRNA and this current was inhibited by HC-067047 in a similar manner to that observed in non-transfected cells (Figure 3B). In contrast, eugenol did not alter I_{Cat} and HC-067047 did not alter I_{Cat} when applied in the presence of eugenol in TRPV4 knockdown cells (Figure 3E). Collectively, these data indicate

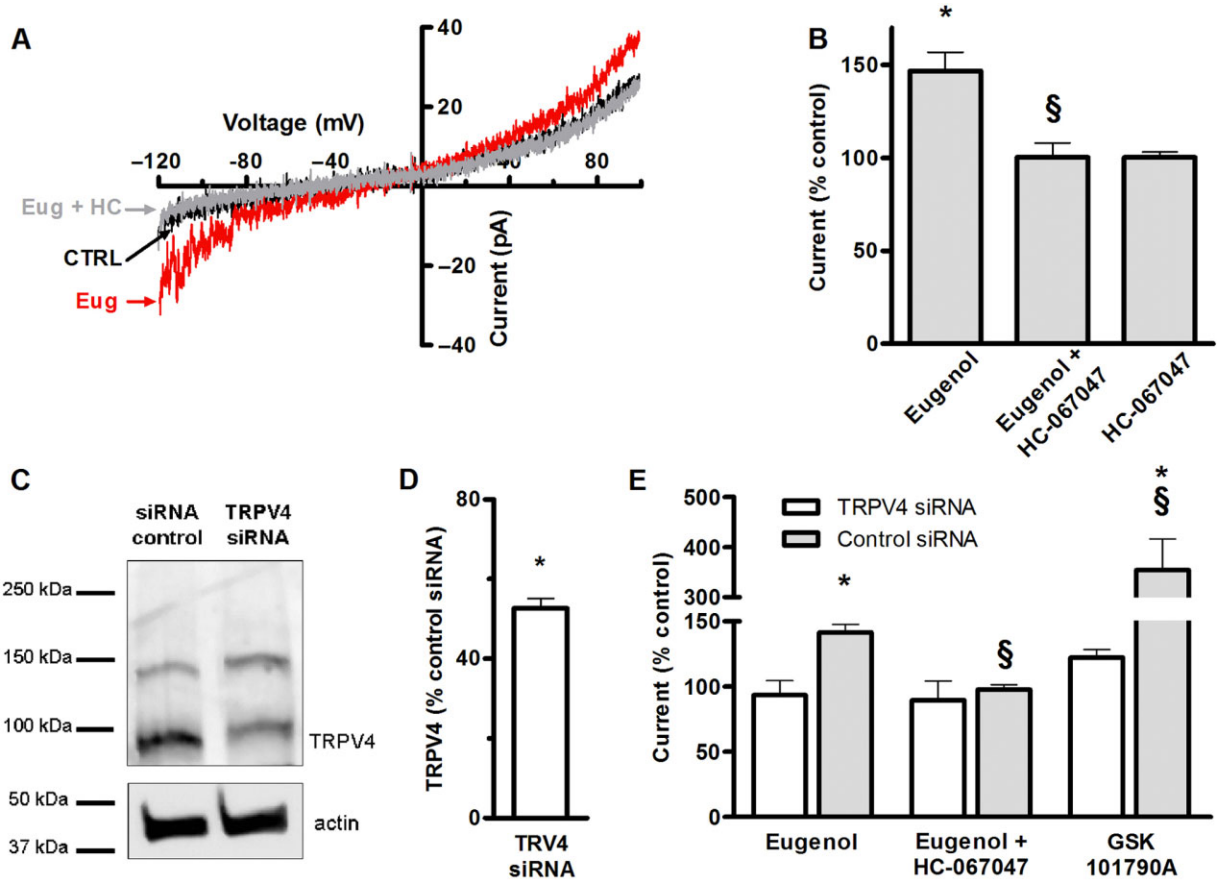

Figure 3

Eugenol activates TRPV4 currents in mesenteric artery endothelial cells. (A) Representative recordings from the same cell illustrating I_{Cat} elicited by voltage ramps from −120 to +100 mV in control conditions, steady-state activation by eugenol (100 μM) before and after the addition of HC-067047 (100 nM). (B) Mean data illustrating the peak I_{Cat} at −100 mV in control conditions (n = 6), and in the presence of eugenol (100 μM, n = 8), eugenol + HC-067047 (100 nM, n = 8), and HC-067047 (100 nM, n = 7). *,§P < 0.05 versus control and eugenol respectively. (C) Representative blots illustrating both TRPV4 and actin total protein in mesenteric artery endothelial cells treated with control siRNA and TRPV4 siRNA. (D) Mean densitometry data of TRPV4 total protein in endothelial cells treated with TRPV4 siRNA (n = 5) versus control (n = 5). *P < 0.05 versus control. (E) Mean data illustrating I_{Cat} peak at −100 mV in cells treated with control siRNA (n = 5) and TRPV4 siRNA (n = 5), exposed to eugenol (100 μM), eugenol + HC-067047 (100 nM) or GSK101790A (10 nM). *,§P < 0.05 versus control and eugenol respectively.

that eugenol activates TRPV4-mediated currents in mesenteric artery endothelial cells.

Eugenol reduces systemic BP via TRPV4 channel activation

The regulation of systemic BP by eugenol was studied *in vivo* in anaesthetized rats. An i.v. injection of eugenol caused a biphasic response composed of an immediate transient reduction in BP and a prolonged hypotension (Figure 4A). The first component peaked ~10 s after eugenol injection and returned to baseline within 1 min. The second prolonged component was initiated ~5 min after eugenol injection and was maintained for ~25 min (Figure 4A).

Specifically, mean control MAP was 91.4 ± 3.1 mmHg (Figure 4C). The eugenol injection almost immediately reduced MAP to 63.4 ± 5.8 mmHg, or to ~68.9% of control (Figure 4A,C). This reduction in BP was associated with bradycardia with a reduction in HR from ~283.9 to 67.3 beats min^{-1}, or to ~23.7 % of control ~5 s after eugenol injection. HR and MAP returned to pre-eugenol levels after 10 s and ~1 min respectively Figure 4C,D). The sustained second component of hypotension plateaued ~20 min after eugenol injection to ~84.1% of control (Figure 4C). HC-067047 injection alone (i.v.) did not alter MAP 10 s or 5 min after application (100.8 ± 1.0 and 101.7 ± 1.6% of control, respectively, n = 5, P > 0.05). Similarly, HR was unaltered 5 s and 5 min after HC-067047 injection (101.1 ± 1.8 and 101.2 ± 0.4% of control, respectively, n = 5, P > 0.05). HC-067047 did not alter the initial eugenol-induced transient reduction in MAP and HR, which were to ~65.8 and 19.7% of control respectively (Figure 4C,D). In contrast, HC-067047 abolished the sustained reduction in BP induced by eugenol (Figure 4B,C). It was shown that the alterations in MAP and HR induced by eugenol were not caused by the vehicle. Ten seconds and 20 min after vehicle injection, MAPs were 101.0 ± 5.4 and 103.0 ± 0.7% of control respectively (n = 5 and P > 0.05 for each). Also the vehicle alone did not alter HR, which was 101.1 ± 0.5 % of control 5 s after injection. These data indicate that eugenol produces both acute bradycardia and an acute and chronic reduction in systemic BP. The data also

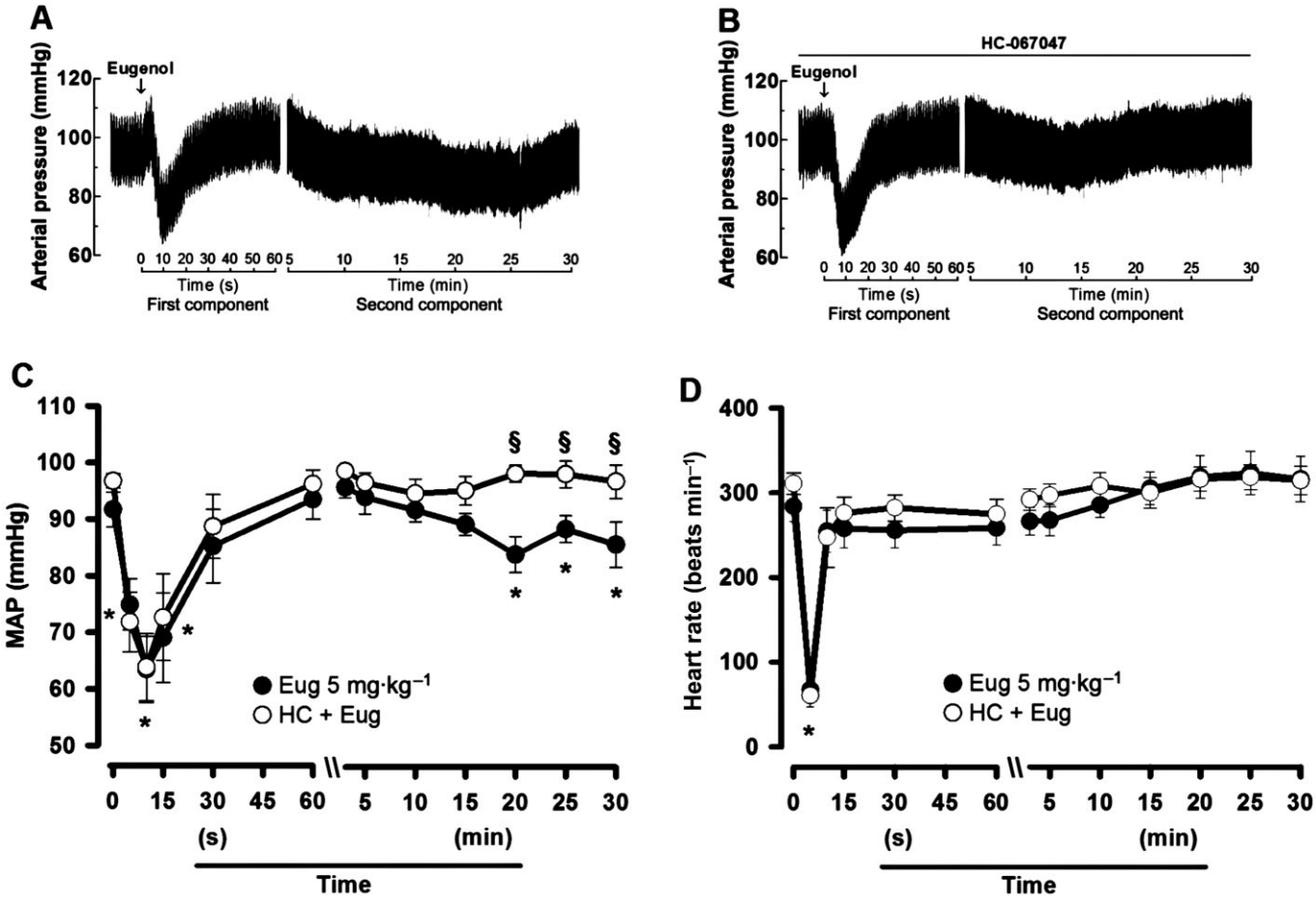

Figure 4

Eugenol reduces systemic BP by activating TRPV4 channels. (A) Representative recording illustrating the effects of eugenol (5 mg·kg^{-1}, i.v.) on BP. (B) Representative trace showing that HC-067047 (9 µg·kg^{-1}, i.v.) attenuates the prolonged reduction in BP induced by eugenol (5 mg·kg^{-1}, i.v.). (C) Mean data for the effects of eugenol (5 mg·kg^{-1}, i.v.) on MAP in the absence (n = 5) or presence (n = 5) of HC-067047 (9 µg·kg^{-1}, i.v.). *§P < 0.05 versus control and Eug 5 mg·kg^{-1} respectively. (D) Mean data for effects of eugenol (5 mg·kg^{-1}, i.v.) on heart rate in the absence (n = 5) or presence (n = 5) of HC-067047 (9 µg·kg^{-1}, i.v.). *P < 0.05 versus control.

indicate that TRPV4 activation contributes to the second chronic component of eugenol-induced hypotension.

Discussion

Here, we investigated the endothelial-dependent vasodilatation evoked by eugenol in rat mesenteric arteries, the mechanisms involved and its functional significance. The major findings were that: (i) endothelium denudation attenuates eugenol-induced vasorelaxation in mesenteric arteries; (ii) relaxation induced by eugenol is reduced by NOS, TRPV4, IK_{Ca} and SK_{Ca} inhibitors, but not by a COX inhibitor; (iii) eugenol activates TRPV4-mediated currents in mesenteric artery endothelial cells; and (iv) eugenol reduced systemic BP via TRPV4 channel activation. These results indicate that eugenol activates TRPV4 channels in endothelial cells, leading to vasodilatation, and a reduction in systemic BP.

Endothelial cells communicate with smooth muscle cells to control contractility, and are, thereby, involved in the regulation of blood flow and BP. A defect in the function of endothelial cells, which can occur in response to abnormal conditions such as ageing and metabolic disease, is a hallmark of vascular pathology and a predictor of major cardiovascular events (Vanhoutte *et al.*, 2009; Davel *et al.*, 2011). The function of endothelial cells is controlled by numerous physiological and exogenous molecules in the circulation, including medicines and dietary derivatives such as eugenol. Our data indicate that eugenol-induced relaxation in mesenteric arteries is mediated, in part, via the endothelium. These results agree with previous studies demonstrating that eugenol-induced vasorelaxation is partially dependent on the endothelium in rat aorta, a conduit vessel, and a whole mesentery preparation (Criddle *et al.*, 2003; Damiani *et al.*, 2003). Eugenol also relaxed endothelium-denuded arteries. One explanation for this effect is that eugenol blocks voltage-dependent Ca^{2+} channels in arterial smooth muscle cells to induce vasodilatation (Peixoto-Neves *et al.*, 2014). In the present study, HC-067047, a selective TRPV4 channel blocker, attenuated eugenol-induced relaxation in endothelium-denuded arteries, suggesting that TRPV4 channels in cells other than endothelial cells are involved in this response. Activation of TRPV4 channels in arterial smooth muscle cells stimulates BK channels, leading to membrane hyperpolarization and vasodilatation, providing one explanation for this result (Earley *et al.*, 2005). Eugenol inhibited TMEM16A, a Ca^{2+}-activated chloride channel, in communication-deficient rat thyroid-derived cell line cells expressing recombinant human TMEM16A (Yao *et al.*, 2012). Eugenol also inhibited the ileal contraction induced by E_{act}, a TMEM16A channel activator (Yao *et al.*, 2012). TMEM16A channel activation leads to membrane depolarization and contraction of arterial smooth muscle cells (Bulley *et al.*, 2012; Dam *et al.*, 2014). Conceivably, an inhibitory effect on TMEM16A channels in smooth muscle cells may also contribute to the eugenol-induced relaxation observed in the present study.

We hypothesized that the activation of TRPV4 channels and downstream signalling pathways in endothelial cells underlies eugenol-induced vasorelaxation. TRPV4 is expressed in rat mesenteric artery endothelial cells (Bagher *et al.*, 2012; Ma *et al.*, 2013). Endothelium-derived NO, EDHF and PGI_2 have been shown to relax mesenteric arteries (Mulvany and Aalkjaer, 1990). Also TRPV4 activation leads to endothelium-dependent vasodilatation via NO and EDHF release (Vriens *et al.*, 2005; Kohler *et al.*, 2006; Saliez *et al.*, 2008; Earley *et al.*, 2009; Zhang *et al.*, 2009; Mendoza *et al.*, 2010). TRPV4 channels generate local Ca^{2+} signals, which activate IK_{Ca} and SK_{Ca} in endothelial cells to induce hyperpolarization, leading to vasodilatation (Sonkusare *et al.*, 2012). Studies have also suggested that the activation of IK_{Ca} and SK_{Ca} is associated with NOS activation and the production of NO (Sheng and Braun, 2007; Dalsgaard *et al.*, 2010; Stankevicius *et al.*, 2011). Furthermore, NOS, SK_{Ca} and TRPV4 channels are located in caveolae, which suggests their close spatial proximity and functional interaction (Sbaa *et al.*, 2005; Absi *et al.*, 2007; Saliez *et al.*, 2008; Michel and Vanhoutte, 2010).

In the present study, eugenol-induced vasorelaxation was attenuated by HC-067047 in endothelium-intact arteries. HC067047 attenuated relaxation induced by GSK1016790A, a TRPV4 agonist, in endothelium-intact arteries, suggesting this blocker is selective for TRPV4 channels (Sukumaran *et al.*, 2013; Zhang *et al.*, 2013). We showed that L-NAME or a combination of TRAM-34 and apamin attenuated eugenol-induced relaxation. Furthermore, the combined inhibition of TRPV4 channels and eNOS or IK_{Ca}/SK_{Ca} did not further reduce the eugenol-induced relaxation response. In contrast, indomethacin did not alter eugenol-induced vasorelaxation. These data indicate that eugenol-induced vasorelaxation involves TRPV4 channel activation in mesenteric artery endothelial cells. We also consider it reasonable to propose that eugenol-induced dilatation occurs as a result of activation of TRPV4-mediated downstream $NOS/IK_{Ca}/SK_{Ca}$ signalling pathways, as proposed by others (Kohler *et al.*, 2006; Mendoza *et al.*, 2010; Sonkusare *et al.*, 2012).

In the present study, indomethacin, TRAM 34/apamin, and HC067047 each caused a small relaxation of U46619-contracted arteries. In contrast, L-NAME increased the tone of the precontracted arteries. Previous studies have demonstrated a similar effect for indomethacin, which attenuated phenylephrine-induced contractions and myogenic tone, demonstrating the involvement of prostanoids, such as thromboxane A_2 and PGE_2, in the regulation of the arterial contractility (Jarajapu *et al.*, 2004; Aloysius *et al.*, 2012). HC-067047 and TRAM-34/apamin were also demonstrated to reduce tension in pulmonary and cerebral arteries, which was associated with off-target effects, probably reducing Ca^{2+} entry to vascular smooth muscle cells (McNeish *et al.*, 2010; Xia *et al.*, 2013).

TRPV4 agonists, such as GSK1016790A, have been shown to damage endothelial cells (Bagher *et al.*, 2012; Sonkusare *et al.*, 2012). Here, repetitive eugenol applications produced relaxant responses of similar amplitude, indicating that endothelial cell damage did not occur. We showed that eugenol produced a smaller TRPV4 current than GSK1016790A at concentrations typically used to study the functional involvement of TRPV4 channels. Thus, eugenol is likely to produce a smaller elevation in intracellular Ca^{2+} concentration than GSK1016790A, which may explain the lack of damage to endothelial cells.

Eugenol increased HC-067047-sensitive I_{Cat} in mesenteric artery endothelial cells. In corroboration of these data,

eugenol did not alter I_{Cat} in endothelial cells subjected to TRPV4 knockdown. As a positive control, GSK1016790A stimulated currents in control siRNA cells, but not in TRPV4 knockdown cells, demonstrating that I_{Cat} were functional after transfection and TRPV4 channels were knocked down by TRPV4 siRNA. Taken together, these data indicate that eugenol activates TRPV4 channels in mesenteric artery endothelial cells to induce vasorelaxation.

In the present study, i.v. administration of eugenol reduced both BP and heart rate. The reduction in BP consisted of two components; the first component is likely to be associated with the reduction in heart rate as these events were temporally aligned. Eugenol inhibits L-type Ca^{2+} channels in canine cardiomyocytes and induces a negative inotropic effect in rat left ventricle papillary muscle by inhibiting extracellular Ca^{2+} influx, providing one explanation for this observation (Damiani *et al.*, 2004; Magyar *et al.*, 2004). It has also been suggested that eugenol-induced bradycardia and hypotension are independent events (Lahlou *et al.*, 2004). The bradycardia was attenuated in rats subjected to vagotomy and by hexamethonium and methylatropine, blockers of nicotinic and muscarinic receptors, respectively, suggesting bradycardia may be of vagal origin (Lahlou *et al.*, 2004). Our data suggest that the second prolonged eugenol-induced reduction in BP is due to vasodilatation, as there was no concomitant alteration in heart rate during this phase, and this effect was attenuated by HC-067047. Providing further support for our observation, 4α-phorbol 12, 13-didecanoate, a TRPV4 channel agonist, reduces BP in part via K_{Ca} channel activation in resistance arteries and by the release of calcitonin gene-related peptide from sensory neurons (Gao and Wang, 2010). These results support the concept that eugenol-induced TRPV4 channel activation leads to vasorelaxation and a reduction in systemic BP *in vivo*.

It is appropriate to compare the potency of eugenol-induced smooth muscle cell relaxation in mesenteric arteries with that in other tissues, although different experimental conditions used among studies make such a comparison exploratory. Our results indicate an EC_{50} for eugenol of ~48 μM in small mesenteric arteries. In rat endothelium-intact cerebral arteries and aorta, eugenol induced relaxation with EC_{50}s of ~235 and ~140 μM respectively (Damiani *et al.*, 2003; Peixoto-Neves *et al.*, 2014). In tracheal smooth muscle, the eugenol EC_{50} was ~570 μM (Lima *et al.*, 2011). Although these studies were performed in a small number of smooth muscle containing tissues, the potency of eugenol appears to be higher in resistance size systemic arteries. Similarly, eugenol appears to be a better relaxant of vasculature than airway, which may be therapeutically beneficial for reducing BP in patients while limiting any side effects.

Our data suggest that eugenol is a more effective activator of TRPV4 than other TRPV channel family members. Eugenol activated an inward current with an EC_{50} of 0.73 mM that was partially reduced by capsazepine, a TRPV1 blocker, in dorsal root ganglia (Ohkubo and Kitamura, 1997). In HEK cells expressing recombinant TRPV1 channels and trigeminal ganglion neurons, 1 mM eugenol activated inward currents, which were blocked by capsazepine and ruthenium red (Yang *et al.*, 2003). In keratinocytes and HEK cells expressing recombinant mTRPV3 and hTRPV3 channels, 2 mM eugenol activated currents (Xu *et al.*, 2006). In the present study, endothelium denudation increased the EC_{50} of eugenol-induced relaxation from ~48 to 190 μM. HC-067047 reduced eugenol-induced relaxation by ~54%. At a concentration of 100 μM, eugenol also activated I_{Cat} in endothelial cells and this effect was inhibited by HC-067047. Collectively, these studies suggest that eugenol is more selective for TRPV4 channels than other TRPV family members.

In summary, our study demonstrates for the first time that eugenol-induced vasorelaxation occurs via TRPV4 channel activation in endothelial cells of resistance arteries. We also showed that eugenol reduces systemic BP via TRPV4 channel activation. Eugenol may be therapeutically useful as an antihypertensive agent and appears to be a viable molecular candidate from which to develop more potent TRPV4 channel activators that have the ability to reduce BP.

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Author contributions

D. P.-N. and Q. W. performed the research. J. H. J. designed the research study. L. V. R. and J. H. L.-C. contributed essential reagents or tools. D. P.-N. and J. H. J. analysed the data. D. P.-N., J. H. J., J. H. L.-C. wrote the paper.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Regulation of U46619-induced contraction by endothelial signalling inhibitors. Indomethacin (10 μM, n = 6), L-NAME (100 μM, n = 5), HC-067047 (100 nM, n = 6), HC-067047 plus L-NAME (n = 5), TRAM-34 plus apamin (1 μM each, n = 6), or HC-067047 plus TRAM-34/apamin (n = 6). *P < 0.05 versus control.

Table S1 Relaxation rate (τ) of eugenol-induced relaxation alone and in presence of L-NAME (100 μM), HC-067047 (100 nM), HC-067047 plus L-NAME, TRAM-34 plus apamin (1 μM each), or HC-067047 plus TRAM-34/apamin in the same arteries.