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RESEARCH PAPER

L-Tryptophan ethyl ester dilates small mesenteric arteries by inhibition of voltage-operated calcium channels in smooth muscle

Ashok Jadhav[1]*, Wenbin Liang[2]*, John Balsevich[3], Guillaume Bastin[2], Jeff Kroetsch[2], Scott Heximer[2], Peter H Backx[2] and Venkat Gopalakrishnan[1]

[1]*Department of Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, Sask, Canada,* [2]*Departments of Physiology and Medicine, University of Toronto, Toronto, Ontario, Canada, and* [3]*Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Sask, Canada*

Correspondence
Venkat Gopalakrishnan, Department of Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada. E-mail: venkat.gopal@usask.ca

*Authors who made equal contributions.

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BACKGROUND AND PURPOSE

L-tryptophan (L-W) is a precursor of the vasoconstrictor, 5-HT. However, acute administration of L-W ethyl ester (L-Wee) lowered blood pressure. The mechanism of action is unknown. This study compares the vascular effects of L-W and L-Wee in intact animals, isolated aortic rings, small mesenteric arteries (MA) and explores possible mechanisms by studies in vascular smooth muscle cells (VSMC) of MA.

EXPERIMENTAL APPROACH

Effects of L-W or L-Wee (5–50 mg kg^{-1}, i.v.) on mean arterial pressure (MAP) and heart rate (HR) were determined in male Sprague-Dawley rats. The effects of L-W and L-Wee on basal tone and of phenylephrine- or KCl-induced contractions of aortic and MA rings were assessed. Effects of L-Wee and L-W on voltage-operated calcium channels (VOCC) of VSMC of MA were also examined in patch-clamp studies.

KEY RESULTS

Administration of L-Wee, but not L-W, evoked a rapid and transient dose-dependent decrease in MAP and HR. While both agents failed to affect basal tone, L-Wee decreased, concentration-dependently, (I_{max} > 98%) tension responses to phenylephrine and KCl in an endothelium-independent manner in aorta (IC_{50} 2 mM) and MA (IC_{50} 17 μM). L-Wee evoked concentration-dependent inhibition of VOCC currents (IC_{50} 12 μM; I_{max} 90%) in VSMC of MA.

CONCLUSIONS AND IMPLICATIONS

Esterified L-W (L-Wee), but not L-W, preferentially relaxed resistance vessels rather than conduit vessels. These effects were associated with blockade of VOCC by L-Wee. Our findings suggest that the falls in MAP and HR induced by L-Wee were due to blockade of VOCC by L-Wee.

Abbreviations

BPM, beats per minute; HR, heart rate; HRW, histidine-arginine-tryptophan; MAP, mean arterial pressure; L-W, L-tryptophan; L-Wee, ethyl ester of L-tryptophan; MA, third-order branches of superior mesenteric artery; VOCC, voltage-operated Ca^{2+} channels; VSMC, vascular smooth muscle cells; W-H, tryptophan-histidine

Introduction

Recent studies suggest that amino acids may regulate blood pressure. The potential health benefits of taurine as a nutritional factor in reducing hypertension and cardiovascular disease have been discussed (Xu *et al.*, 2010; Yamori *et al.*, 2009) and dietary intake of vegetable proteins rich in glutamic acid is known to lower the incidence of essential hypertension (Stamler *et al.*, 2009). Acute intravenous administrations of L-serine and glycine lowered blood pressure (BP) in normotensive rats and *in vitro* addition of L-serine evoked endothelium-dependent vasodilatation of third-order branches of rat superior mesenteric artery (MA) (Mishra *et al.*, 2008a,b). Moreover, food-derived bioactive peptides have the potential to reduce cardiovascular risk and lower BP (Erdmann *et al.*, 2008). *In vitro* addition of a dipeptide (tryptophan-histidine [W-H]) and a tripeptide (histidine-arginine-tryptophan [HRW]) containing L-tryptophan (L-W) caused endothelium-independent vasodilatation with IC_{50} values in concentration ranges of 1–3 mM in KCl-depolarized rat thoracic aortic rings. However, addition of a mixture of these amino acids in similar concentration ranges in organ baths failed to cause any vasodilatation. It was also suggested that L-W-containing peptides, but not the constituent amino acids, blocked voltage-operated Ca^{2+} channels (VOCCs) (Tanaka *et al.*, 2008; 2009; Wang *et al.*, 2010b; channel nomenclature follows Alexander *et al.*, 2011.

L-W, an essential amino acid, is the precursor of 5-HT, a potent endogenous vasoconstrictor and a central neurotransmitter. L-W is present in the circulation and arterial tissue such as rat aorta can biosynthesize 5-HT from L-W (Ni *et al.*, 2008). Despite the possibility that L-W administration could result in 5-HT-mediated vasoconstriction, both acute and chronic administrations of cell-permeant L-W ester analogues reduced mean arterial pressure (MAP) in hypertensive rat models (Sved *et al.*, 1982; Safdy *et al.*, 1982; Wolf and Kuhn, 1984a,b; Fregly *et al.*, 1989; Pop *et al.*, 1990). These effects of L-W analogues have been attributed to increased generation of 5-HT and other biogenic amines in the CNS while a few studies have argued that changes in peripheral mechanisms could also account for the hypotensive effect of L-W analogues (Wolf and Kuhn, 1984a,b; Fregly *et al.*, 1989; Pop *et al.*, 1990). A modest reduction in MAP was also reported following oral administration of 50 mg kg^{-1} of L-tryptophan ethyl ester (L-Wee) in patients with essential hypertension (Feltkamp *et al.*, 1984). Moreover, chronic administration of 5-HT has been shown to exert a paradoxical fall in MAP in both normotensive and hypertensive rats via increased nitric oxide synthase activity (Diaz *et al.*, 2008; Watts, 2009). However, these studies failed to address the effect of L-W analogues on changes in heart rate (HR). A recent study has shown that addition of L-W and its endothelium-derived metabolite, kynurenine, evoked profound vasodilatation of porcine coronary artery. Kynurenine, but not L-W administration decreased MAP in spontaneously hypertensive rats (Hofmann, 2010; Y Wang *et al.*, 2010).

While it is known that L-W ester analogues exert antihypertensive effects, the mechanisms of this action have not been adequately explored. The plasma level of L-W in overnight-fasted human volunteers is 44 μM (Cynober, 2002) And, in rats, the plasma concentration of L-W varies between 76 and 125 μM (Knott and Curzon, 1972). Apart from absorption from the GI tract, circulating levels of insulin, glucagon, cortisol and thyroid hormones are known to affect plasma levels of amino acids and their movement across cells (Cynober, 2002). At present, it is not known whether an elevation in plasma levels of L-W contributes to altered vascular tone via 5-HT/kynurenine-dependent or -independent mechanisms. In an attempt to address all these issues, we first compared the acute *in vivo* effects of L-W and L-Wee administration on changes in MAP and HR in anaesthetized Sprague–Dawley rats. Second, we carried out parallel *in vitro* studies to compare the effects of L-W and L-Wee on changes in basal tone and alterations in phenylephrine-induced, and KCl-depolarized steady-state constrictor responses in rat aorta, a conduit vessel, and third-order branches of superior MA, that may contribute to the resistance function of circulation. Finally, to support our functional data, mechanistic studies were conducted to examine the effects of both L-W and L-Wee on VOCC currents in single vascular smooth muscle cells (VSMC) freshly dispersed from the MA.

Methods

Animals

All animal care and experimental protocols complied with the current laws governing animal experimentation in the UK and were approved by the respective Animal Care Committees of both Universities. Male Sprague–Dawley rats (12 week-old) were purchased from Charles River Laboratories (St. Constant, QC, Canada) and maintained at a controlled temperature (23 ± 2°C) under a regular light–dark cycle (light period 0700–1900 h) with free access to food and water. They were allowed to acclimatize for about a week. The experiments were performed when the animals were 13 weeks old and were weighing between 300 and 350 g. The rats ($n = 40$) were anaesthetized with an intraperitoneal injection of thiopental sodium (100 mg kg^{-1}, dissolved in saline at 25 mg mL^{-1}) and used for *in vivo* studies or exsanguinated to isolate blood vessels for *in vitro* studies.

Measurement of BP and HR

The anaesthetized rats were placed on a heating pad to maintain the temperature at 37°C (measured by a rectal probe) and were allowed to stabilize and breathe spontaneously through a tracheal cannula. The right carotid artery and the left femoral vein were cannulated with polythene cannulas. The carotid cannula (i.d. 0.4 mm and o.d. 0.8 mm) was filled with heparinized saline (50 U·mL^{-1}) and was connected to a pressure transducer to record the MAP and HR (beats per min) using the Powerlab data acquisition system (AD Instruments Pvt. Ltd, Sydney, Australia). The anaesthesia lasted throughout the duration of the 2 h experiment. Thiopental administration did not cause a significant depression of BP or HR. Supplemental doses of thiopental (20 mg kg^{-1}, if required) were given through the i.v. cannula and the experiments were continued only if the basal MAP and HR values had stabilized to the original level. The detailed methodology for the measurement of BP and HR were described earlier (Laight *et al.*, 2000; Kawabata *et al.*, 2003; Shinde *et al.*, 2005; Desai

et al., 2006; Mishra *et al.*, 2010). The femoral vein cannula (i.d. 0.5, o.d. 0.63 mm) was used to administer either L-W (5–50 mg·kg^{-1}) or L-Wee (5–50 mg·kg^{-1}) as bolus injections (0.3 mL·kg^{-1}) after a steady-state BP and HR levels were maintained. The total volume of either L-W or L-Wee administered to each rat was kept to a minimum (<1.2 mL) over a 60 min period and no more than five doses of each agent was given to each rat. Enough time was allowed between responses for the MAP to recover to the basal level following each administration.

Rat isolated thoracic aorta

The effects of L-W and L-Wee on changes in tone of rat isolated thoracic aortic rings were determined in organ baths containing 10 mL Krebs buffer (in mM: 120, NaCl; 4.8, KCl; 1.2, $MgCl_2$; 1.8, $CaCl_2$; 1.2, KH_2PO_4; 25, $NaHCO_3$; 11, glucose; pH 7.4 gassed with 95% O_2, 5% CO_2 at 37°C) maintained under a resting preload tension of 2 g as described previously (Hopfner *et al.*, 1998). Adequate care was taken to insert the hooks without damaging the endothelium. The aortic rings were first contracted with a submaximal concentration (~EC_{80} level) of selective α_1-adrenoceptor agonist, phenylephrine (1 μM). Once the steady-state tonic response was attained, the tissues were washed in normal Krebs buffer for a period of 1 h and the response to the same concentration of phenylephrine was repeated to ascertain that the sustained tonic response to phenylephrine was reproduced. Then, in the presence of steady-state tonic response to phenylephrine, a fixed concentration of ACh (10 μM) was added to assess the extent of vasodilatation in phenylephrine-constricted rings. If vasodilatation to ACh was >90%, it was considered as an endothelium-intact preparation. In some studies, endothelium was removed by gently rubbing the intimal surface of aortic rings with a cotton swab wetted with Krebs buffer before the tissues were mounted. The endothelium was considered as removed when the vasodilator response to ACh (10 μM) was reduced to ≤10% of its original level but with no change in the vasodilator response to SNP (10 μM). Once a steady tonic response was reached following the addition of phenylephrine, cumulatively increasing concentrations of either L-W (1 μM–8 mM) or L-Wee (1 μM–8 mM) were added in such a way that the next concentration was added only after the response to the previous concentration had plateaued. For studies involving KCl-evoked tension responses, the tissues were washed in Krebs buffer after determining the presence or absence of functional endothelium in PE-constricted state. After the tissues were recovered by repeated washing for a minimum of 1 h, they were challenged with a depolarizing Krebs solution containing 100 mM KCl (with equimolar removal of NaCl), to induce a sustained tonic response. Then, cumulatively increasing concentrations of either L-W (1 μM–8 mM) or L-Wee (1 μM–8 mM) were added. Thus, the changes in isometric tension evoked by L-W or L-Wee in both phenylephrine- and KCl-constricted rings were determined in endothelium-intact and endothelium-denuded vessels of the same rat. The tension responses were recorded (in g) on a chart programme (Chart V4.0.1) using a Powerlab/8SP data acquisition system (AD Instruments Pvt. Ltd, Sydney, Australia) and the values were then converted to mN.

Third-order branches of superior MA

A section of mesentery around 10 cm distal to the pylorus was rapidly removed and placed in ice-cold Krebs (kept oxygenated in 95% O_2, 5% CO_2) with the same composition as described above except that glucose level was 5.5 mM. A lower glucose level in the buffer was used as we adopted the procedure described by earlier studies for this preparation (Mishra *et al.*, 2008a; Thakali *et al.*, 2010). With the aid of a dissection light microscope, the third-order branches of the rat superior MA (i.d. < 250 μm) were carefully isolated (Mishra *et al.*, 2008a; Thakore and Ho, 2011). Four to eight rings (~2 mm length) were isolated and suspended between a micropositioner and force transducer with stainless steel wires (40 μm diameter) in a myograph chamber, model 610 M Multi Wire Myograph System (Danish Myotechnology, Aarhus, Denmark). Resting tension (2 mN) was fixed for initial equilibration period of 1 h (Mulvany and Halpern, 1976; Allen *et al.*, 2002). The changes in force developed were recorded as increase in mN on a Powerlab data acquisition system using the protocol described above for aortic rings. In some experiments, endothelium was removed by scratching the intimal layer by passing a human hair a few times through the isolated arterioles before setting it up in wire myograph apparatus. It was considered as denuded if the dilator response to ACh was ≤10%. The concentration–response relationship for cumulative additions of increasing concentrations of either L-W (0.1 μM–640 μM) or L-Wee (0.1 μM–640 μM) were determined in both endothelium-intact and endothelium-denuded states under resting tension and in phenylephrine- or KCl-constricted states.

Isolation of single VSMC

Rat MA were dissected in ice-cold Hank's balanced salt solution (in mM: 140, NaCl; 4.2, KCl; 1.2, $MgCl_2$; 1.2, KH_2PO_4; 10, HEPES; 6, glucose; pH 7.4 adjusted with NaOH) and stripped of connective tissue under a dissecting microscope. Arteries were placed in Hank's solution at room temperature for 10 min and then transferred into Hank's solution, containing 0.2 mg·mL^{-1} bovine serum albumin (BSA), 1 mg·mL^{-1} papain, and 0.85 mg·mL^{-1} dithiothreitol and kept at 4 °C for 50 min and then at 37°C for 15 min. Arteries were transferred into Hank's solution, containing 1 mg·mL^{-1} BSA, 1 mg·mL^{-1} collagenase type II, and 0.5 mg·mL^{-1} elastase and kept at 37°C for 30 min. Arteries were then washed in fresh Hank's solution at room temperature for 10 min. The 3rd to 5th degree small branches were cut off and put into Hank's solution and single VSMC were released by gentle trituration through a fire-polished Pasteur pipette. Cell suspensions were kept at 4°C and used for experiments within 8 h of dissociation (Liang *et al.*, 2009).

Recording of Ba^{2+} currents through voltage-operated Ca^{2+} channels (VOCC)

Activities of VOCC were measured by recording Ba^{2+} currents from single MA VSMC using whole-cell patch-clamp technique (Axopatch 200B, Axon Instruments, Foster City, CA) at 22°C. VSMC were placed in a perfusion chamber (volume 0.5 mL) on a microscope stage (IX50, Olympus Inc., Center Valley, PA) and superfused with a solution containing (in mM) 137, NaCl; 5.4, CsCl; 1.0, $MgCl_2$; 5.0, $BaCl_2$; 10, HEPES;

and 10, glucose; pH 7.4 adjusted with NaOH. The pipettes had a resistance of 2.0–2.5 MΩ when filled with a solution containing (in mM) 115, CsCl; 20, TEA-Cl; 1.0 $MgCl_2$; 10 EGTA; 10, HEPES; 5 Mg-ATP; and 0.2, Na_2GTP; pH 7.2 adjusted with CsOH. Some of the VSMC were studied with the nystatin-perforated method by adding 200 $\mu g \cdot mL^{-1}$ nystatin to the pipette solution. Series resistance was compensated by 80%–90%. VSMC were held at −60 mV and stepped to −40 mV for 300 ms before voltage steps from −60 to +50 mV for 300 mS to examine L-type VOCC (Liang *et al.*, 2009). Currents at 0 mV were recorded every 10 s during L-W or L-Wee perfusion. The mean ± SEM capacity values of VSMC were 13.6 ± 1.6 pF (n = 8).

Statistical analysis

For *in vivo* studies, the percent (%) fall in MAP and HR attained from basal value following administration of each dose of L-W or L-Wee was determined in five rats and the pooled values are expressed as mean ± SEM (n = 5 rats). The % fall in either the MAP or HR attained for each dose of L-Wee was calculated considering the MAP and HR value before the addition of L-Wee as the control/basal value. The data were analyzed for statistical significance using one-way ANOVA as the same variable (MAP or HR change) was compared in the same animal before and after the additions of L-W or L-Wee, followed by Tukey *post hoc* test and the differences between means was considered significant when the P-value was <0.05. These results were presented as line graphs. The closest P-value obtained is given in the Results section.

For *in vitro* study, experiments were performed each day using both endothelium-intact and endothelium-denuded vessels after they were isolated from one rat and tested for inhibition of tension responses in phenylephrine- or KCl-constricted states. The vasodilator responses observed were normalized as % inhibition of steady-state tonic response (100%) evoked by fixed concentration(s) of either phenylephrine or KCl. As the inhibitory effect reached close to 100%, the IC_{50} and I_{max} values could be generated from each concentration-inhibition response curve using Prism software (Graph Pad Inc., La Jolla, CA). The data obtained using vessels isolated from one rat for a specific condition of incubation (either endothelium-intact or endothelium-denuded using either phenylephrine or KCl as the constrictor agent) were pooled and the mean value from that rat was considered as n = 1. Then, similar experiments were replicated with blood vessels isolated from 7 to 9 rats ($n \geq 7$ rats) for that specific condition. Thus, the final mean ± SEM values shown in the Results section represent the data gathered from several rats. As these studies were performed in two tissues, using two agonists under different incubation conditions, the differences in mean ± SEM values were analysed using two-way ANOVA, followed by Tukey's *post hoc* test. The data were considered significant when the P-value was <0.05. However, for assigning the level of significance, the closest P-value reached was provided in the Results section. The mean ± SEM values obtained in Ba^{2+} current studies (performed at the University of Toronto) were subjected to one-way ANOVA followed by Bonferroni *post hoc* comparisons, as these studies were conducted in the same VSMC before (0 μM) and after perfusion of indicated concentrations of either L-W or L-Wee.

Materials

Acetylcholine chloride, BSA, dithiothreitol, EGTA, nifedipine, phenylephrine hydrochloride, papain, sodium nitroprusside (SNP), L-W and L-Wee, as well as all the salts used in the preparation of Krebs buffer were of analytical grade obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Collagenase type II and elastase were obtained from Worthington Biochemical Inc. (Lakewood, NJ, USA). Thiopental sodium was obtained from Abbott Laboratories Ltd. (Saint-Laurent, QC, Canada).

Results

In vivo *studies*

As shown in Figure 1A, neither the bolus administration of increasing doses of L-W (5–50 $mg \cdot kg^{-1}$, i.v.), nor injection of similar volume of vehicle (i.e. saline) affected the BP or HR in rats. In contrast to L-W, administration of similar doses of L-Wee (5–50 $mg \cdot kg^{-1}$, i.v.) evoked dose-dependent transient decreases in MAP and HR that returned to baseline within 5 min (Figure 1B). Pooled data (n = 5 rats) revealed that the reductions attained in MAP were significant ($P < 0.01$) starting from a dose of 10 mg kg^{-1} and the maximum fall (>45%) was reached at a dose of 30 $mg \cdot kg^{-1}$. The administration of a higher dose of L-Wee (50 $mg \cdot kg^{-1}$) following the recovery of MAP to the basal value did not elicit a further fall in MAP. The fall in HR attained with increasing doses of L-Wee administration was significant only at doses at or above 20 $mg \cdot kg^{-1}$ (Figure 1C).

In vitro *studies using aorta and MA*

To determine whether the *in vivo* effects of L-Wee were related to vasodilatation, we compared the effects of incubation with L-W and L-Wee on tension development in isolated aortae and MA. The addition of cumulatively increasing concentrations of either L-W or L-Wee (1 μM–8 mM) prepared in Krebs buffer failed to affect the basal tone in either aortae or MA (data not shown). Next, we examined the effects of L-W and L-Wee in vessels pre-constricted with either phenylephrine or KCl. Because the steady-state tonic constrictor responses varied between preparations, particularly at the lower concentrations of either phenylephrine or KCl, the effects of L-W and L-Wee on pre-constricted vessels were performed at concentrations of phenylephrine and KCl that produced 80–90% of maximal constrictor response. Thus, the concentrations used were 1 and 10 μM phenylephrine in aortic rings and MA, respectively and 100 mM KCl in both vessels. At these concentrations, the tonic constrictor responses remained sustained for well over 60 min. The tension response (in mN) generated by these levels ($EC_{80\text{-}90}$) of phenylephrine and KCl in both endothelium-intact and endothelium-denuded vessels isolated from rats ($n \geq 7$) are shown in Table 1. The steady tonic responses to phenylephrine and KCl reached were not changed in the same tissue by the loss of endothelium. The tension responses reached in MA rings with either phenylephrine or KCl was significantly lower ($P < 0.01$) compared with the responses in aortic rings for the same constrictor agent (Table 1).

Addition of either Krebs buffer or concentrations of L-W up to 8 mM (prepared in Krebs buffer with adjustments in pH

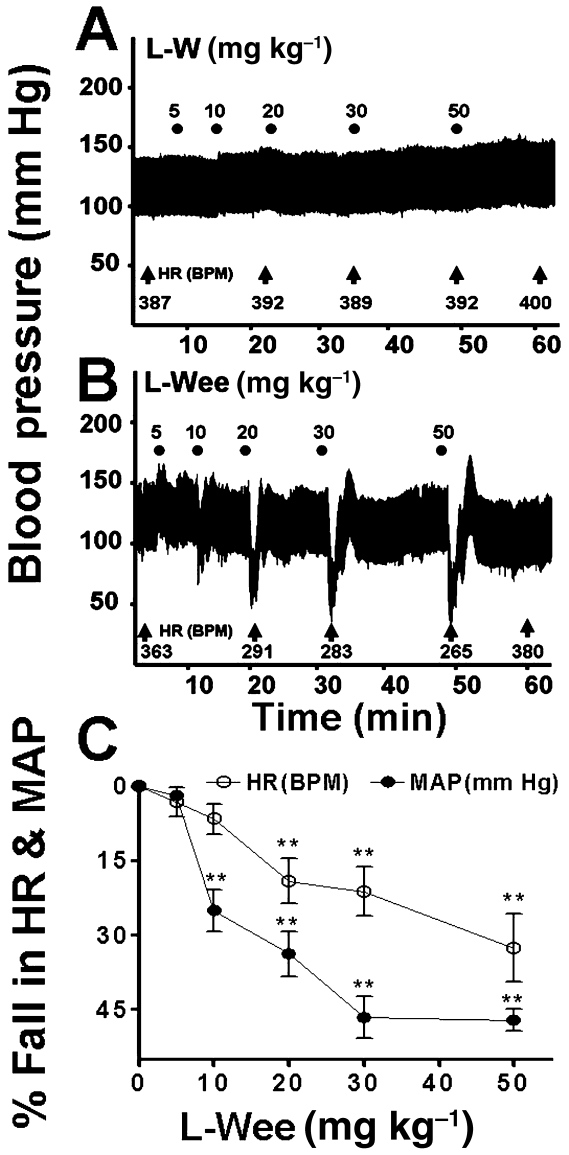

Figure 1

The effects of acute i.v injection of increasing doses of either L-W (5–50 mg kg^{-1}, A) or and ethyl ester of L-W (L-Wee 5–50 mg·kg^{-1}, B) on changes in blood pressure and heart rate (HR–BPM) in 13 week-old male Sprague–Dawley rats, performed in parallel are shown. The changes in HR values determined as beats per minute (BPM) following administration of increasing doses of either L-W or L-Wee are also shown in A and B. The graphs show the mean (± SEM) data (n = 5 rats) for the % fall in mean arterial pressure (MAP, mmHg) or HR (BPM) attained following administration of indicated doses of L-Wee (C). **P < 0.01, significantly different from basal value before the addition of indicated dose of L-Wee.

to 7.4) failed to affect the basal tone or the sustained tonic response generated by either phenylephrine or KCl in both endothelium-intact and endothelium-denuded aortae and MA. Typical responses of endothelium-denuded aortae and MA are shown (Figures 2A, 2B, 3A, 3B). In contrast to L-W, addition of L-Wee (0.1/1 μM–8 mM) led to concentration-dependent reductions in the steady-state constriction by phenylephrine and KCl in endothelium-denuded aortae and MA (Figures 2C, 2D, 3C, 3D). In aortic rings, the fall in tension response evoked by L-Wee was slow and sustained beginning at a concentration of 320 μM and the inhibition was complete at concentration ranges between 4 and 8 mM (Figure 2C and 2D). On the other hand, the responses of MA vessels to L-Wee were transient at lower concentrations with the threshold response beginning at 5 μM while sustained and maximal inhibition occurred at concentration ranges between 80 and 160 μM (Figure 3C and 3D). Using the peak inhibitory response at each concentration, we generated the concentration–inhibition response curves to L-Wee along with the lack of inhibition at similar concentration levels for L-W (Figure 4 A–D). The estimated IC_{50} and I_{max} values established that the MAs were far more sensitive to the inhibitory effect of L-Wee (2 log units lower IC_{50} values, $P < 0.01$) than aortic rings. The presence or absence of endothelium or the vasoconstrictor agent used to elevate the tone (phenylephrine or KCl) did not affect the inhibitory responses (IC_{50} and I_{max} values) to L-Wee (Table 2).

VOCC recording in VSMC of MA

To explore the cellular and ionic mechanisms for the L-Wee-mediated vasodilation, we investigated the effects of L-Wee and L-W on VSMC L-type VOCC, a key regulator of intracellular Ca^{2+} level and contraction. Consistent with its lack of vasodilator action, a high concentration of L-W (100 μM) did not affect VOCC-mediated Ba^{2+} currents recorded in isolated single VSMC of MA (Figure 5A). In contrast, L-Wee (100 μM) inhibited ($P < 0.01$) the Ba^{2+} currents over the voltage range tested (Figure 5B and 5C). The L-Wee sensitive currents showed a current–voltage relationship typical of L-type VOCCs (Figure 5C lower panel). The effects of L-Wee on Ba^{2+} currents were also dose-dependent (Figure 5D). These data suggest that L-Wee inhibits VOCC activity in VSMC of MA, which may underlie its vasodilator effects.

Discussion

Four major findings emerge from the present study. (i) L-Wee but not L-W-evoked endothelium-independent vasodilatation. This finding is supported by the dose-dependent fall in MAP after L-Wee administration in anaesthetized rats. The fall in MAP was not accompanied by an increase in HR following L-Wee administration. (ii) Recent work suggests that L-W could undergo bioconversion to kynurenine in vascular endothelial cells and thus could promote endothelium-dependent vasodilatation (Wang *et al.*, 2010a). Data from the present study do not support the notion that either L-W or its cell-permeant analogue, L-Wee, exerts endothelium-dependent vasodilatation in both conduit-type bold vessel (aorta) and third-order branches of MA. (iii) The endothelium-independent vasodilator responses were evoked by L-Wee at lower concentrations in MA (IC_{50} 12–17 μM) than in aortic rings (1.2–2.2 mM), suggesting a greater inhibition in smaller vessels than in conduit-type vessels. (iv) The patch-clamp study suggests that L-Wee inhibits L-type VOCC in freshly isolated VSMC in the same concentration ranges as seen in functional studies.

Table 1

A comparative analysis of sustained steady-state tonic response elicited by EC_{80-90} concentrations of either phenylephrine (PE) or KCl in endothelium-intact (Endo [+]) and endothelium-denuded (Endo [–]) aortic and third-order branches of superior mesenteric artery (MA) rings of 13 week-old male Sprague–Dawley rats

Preparation	Condition	PE	KCl
Aorta	Endothelium (+)	19.9 ± 2.4 mN	30.7 ± 3.1 mN
	Endothelium (–)	22.7 ± 3.2 mN	33.6 ± 4.9 mN
MA	Endothelium (+)	9.1 ± 1.8 mN**	9.9 ± 2.1 mN**
	Endothelium (–)	10.6 ± 2.1 mN**	10.9 ± 3.2 mN**

The responses to phenylephrine or KCl determined in several vessels from one single rat were pooled to generate one single mean value. These studies were replicated in vessels from different rats to calculate the final mean ± SEM value (n = 7-9) for each condition.
**$P < 0.01$, significantly different from corresponding data for the same agonist and condition in aortic rings.

Table 2

The analysis of concentration-inhibition response curves to L-Wee in phenylephrine (PE)-constricted or KCl-depolarized aortic and third-order branches of superior mesenteric artery (MA) rings determined in endothelium-intact (endothelium [+]) or endothelium-denuded (endothelium [–]) preparations

Tissue	Agonist	Condition	L-Wee – IC_{50}	L-Wee – E_{max} (%)
Aorta	PE (1 μM)	Endothelium (+)	1.2 ± 0.3 mM	100 ± 3
		Endothelium (–)	1.2 ± 0.4 mM	99 ± 2
	KCl (100 mM)	Endothelium (+)	1.7 ± 0.5 mM	100 ± 3
		Endothelium (–)	2.2 ± 0.5 mM	100 ± 2
MA	PE (10 μM)	Endothelium (+)	14.6 ± 2.9 μM**	99 ± 1
		Endothelium (–)	17.8 ± 3.2 μM**	98 ± 3
	KCl (100 mM)	Endothelium (+)	12.0 ± 2.8 μM**	99 ± 1
		Endothelium (–)	16.6 ± 2.9 μM**	100 ± 2

Each data point is a mean ± SEM of vessels isolated from a minimum of seven rats.
**$P < 0.01$, significantly different from corresponding IC_{50} value determined in rat aortic rings for the same incubation condition. The IC_{50} and I_{max} values for L-Wee compared were subjected to two-way ANOVA given the two tissue variables and two different concentrations of phenylephrine were employed, although they produced EC_{80-90} effect in either tissue.
Note: The presence or the absence of the endothelium did not affect the IC_{50} and I_{max} value for L-Wee determined against either phenylephrine- or KCl-evoked responses in the same vessel.

Earlier studies had failed to address the mechanism for the antihypertensive effect of L-W analogues (see Pop *et al.*, 1990). A recent paper proposed that L-W reduced the tonic vasoconstrictor responses to a thromboxane agonist, U-46619, in porcine coronary artery in an endothelium-dependent manner, because of bioconversion of L-W to kynurenine. Kynurenine is a potent endothelium-derived vasodilator that promotes increased cAMP turnover in VSMC via activation of adenylate cyclase. Treatment with kynurenine but not L-W-reduced MAP in spontaneously hypertensive rats (Hofmann, 2010; Wang *et al.*, 2010a). In our studies, incubation with L-Wee attenuated the steady-state tonic responses in endothelium-denuded vessels with closely similar IC_{50} values against both phenylephrine and KCl in aortae and MA. On the other hand, addition of high concentrations of L-W alone failed to show vasodilatation in both sets of blood vessels. These observations suggest that L-Wee exerts its actions independent of its bioconversion to kynurenine, via its effects on VSMC.

Consistent with earlier stated details, the IC_{50} values for L-Wee against both phenylephrine- and KCl-evoked responses were closely similar in endothelium-intact as well as endothelium-denuded aortae or MA. However, the inhibitory effect of L-Wee was more sensitive in small MA (Table 2). The conclusion that L-Wee exerted its effects on arterial vessels via its actions on VSMC isolated from MA is also consistent with our results showing that L-Wee was able to block L-type VOCC at concentration ranges similar to those required for the relaxation of pre-constricted MA. One observation that is not readily explained by the conclusion that L-Wee relaxes vessels via L-type VOCC inhibition in VSMC is the observation of differences in sensitivity between resistance-type and conduit vessels to L-Wee. While it is conceivable that L-type VOCCs in small blood vessels (resistance

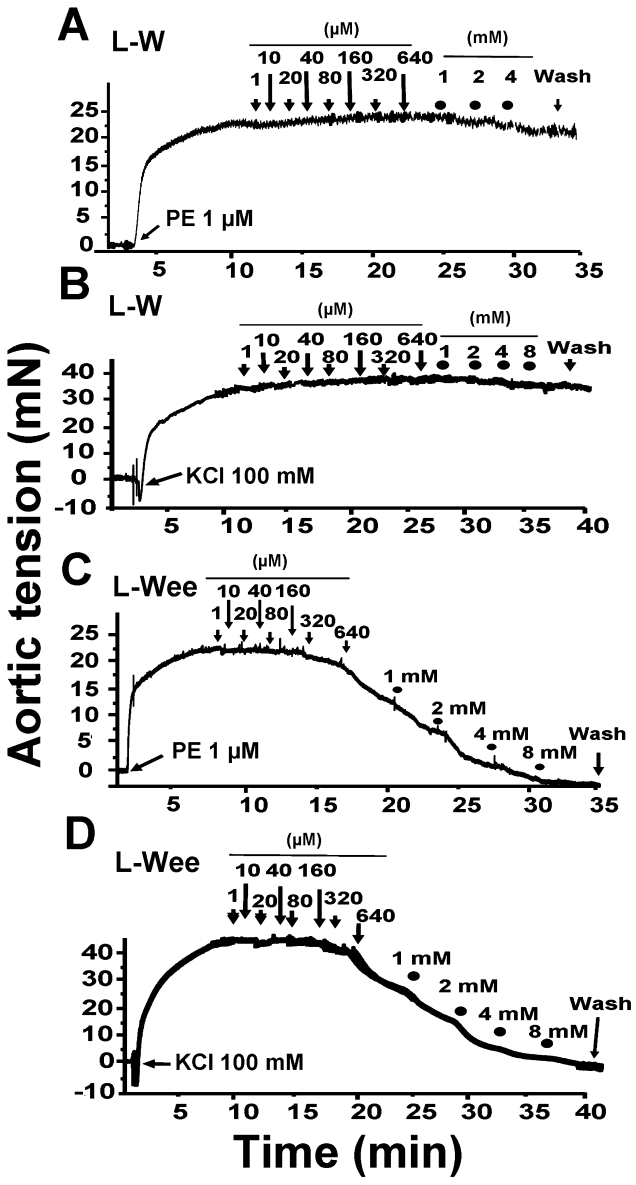

Figure 2

Experimental records showing the lack of changes in the steady-state tonic response to cumulative addition of increasing concentrations of L-W (1 μM–8 mM) in endothelium-denuded rat aortic rings that were constricted by a fixed concentration of either the α_1-adrenoceptor selective agonist, phenylephrine (PE; 1 μM; A), or KCl (100 mM; B). The responses to addition of L-Wee (1 μM–8 mM) in parallel endothelial-denuded rings that were constricted with either phenylephrine (1 μM, C) or KCl (100 mM, D) are shown. Similar patterns of responses were obtained in aortic rings isolated from nine rats.

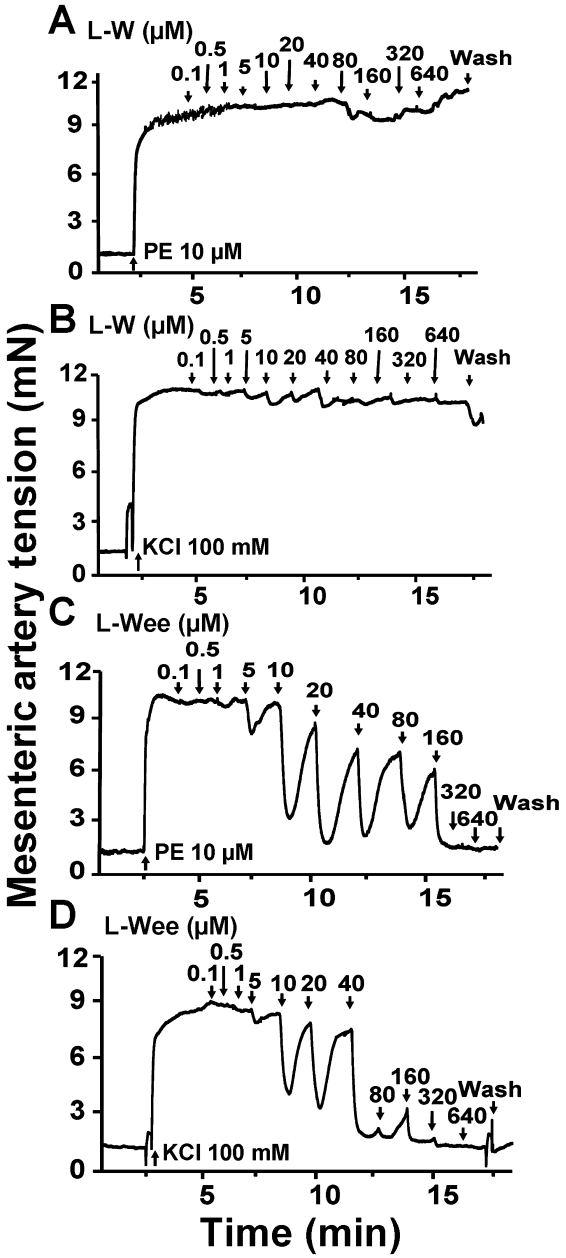

Figure 3

Experimental records showing the lack of changes in the sustained tonic response to cumulative addition of increasing concentrations of L-W (0.1 μM–640 μM) in endothelium-denuded third-order branches of rat superior MA that were constricted by either phenylephrine (PE; 10 μM; A) or KCl (100 mM, B). The responses to addition of L-Wee (0.1 μM–640 μM) in a parallel endothelial-denuded MA constricted with either phenylephrine (10 μM, C) or KCl (100 mM, D) are shown. Similar response patterns were obtained in vessels isolated from eight rats.

type) differ from those in conduit vessels, it is more likely that these differences originate from differences in drug delivery or metabolism of the esterified L-W in VSMC of these two vessel types. Specifically, the inhibitory effects of L-Wee were much lower and sustained in both endothelium-intact or endothelium-denuded rat aortic rings (IC_{50} 1.2–2.2 mM) in comparison with the rapid and transient inhibition at much lower concentrations of L-Wee in MA (IC_{50} 12–17 μM). These observations suggest that L-Wee, an ester of L-W, is rapidly degraded after its entry into VSMC of conduit-type blood vessels compared with small MA blood vessels. Moreover, the rapid recovery of tension response following the application

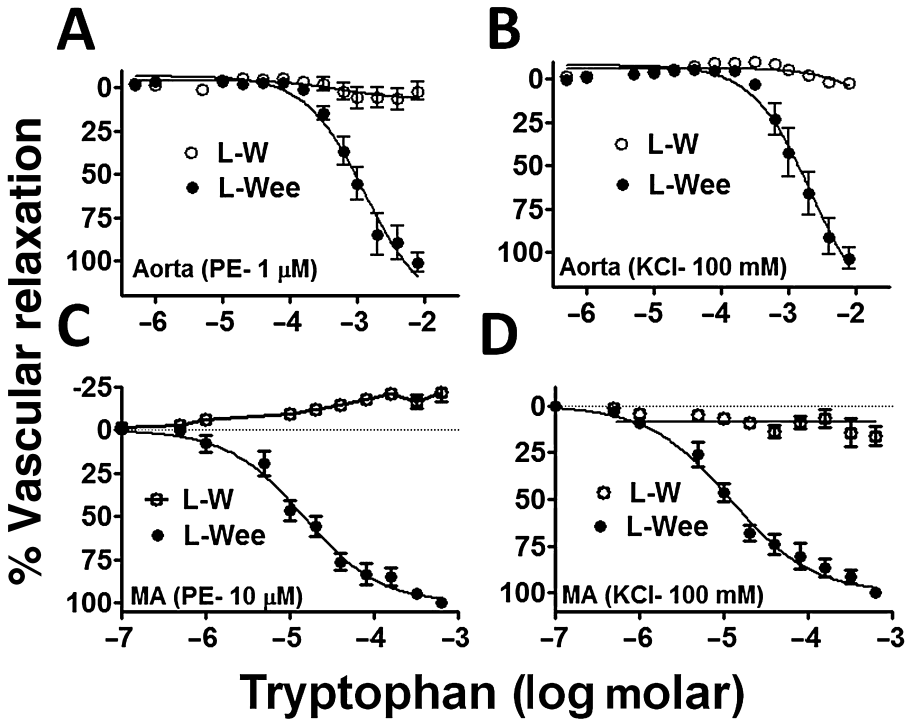

Figure 4

The comparison of concentration–inhibition response curves to L-W and L-Wee in endothelium-denuded aortic rings (A, B) and MA (C, D) that were constricted with a fixed concentration of either phenylephrine (PE; 1 or 10 μM) or KCl (100 mM) after their isolation from 13 week-old male Sprague–Dawley rats. Each data point is mean ± SEM value obtained from vessels isolated from a minimum of eight rats ($n \geq 8$ rats).

of lower concentration of L-Wee in isolated MA may suggest that L-Wee induces feedback activation of enzymes involved either in L-Wee metabolism or its extrusion from the cells. Clearly, further studies will be required to assess the basis for the differential responses of the resistance versus conduit vessels to relaxation induced by L-Wee.

The notion that the de-esterified L-Wee might be rapidly metabolized in target cells like VSMC, is also supported by our observation that acute administration of bolus doses of L-Wee causes transient reductions in MAP. This finding is consistent with previous reports that esters of L-W cause reductions in BP (Safdy *et al.*, 1982; Pop *et al.*, 1990). Interestingly, the acute fall in MAP induced by L-Wee did not lead to an expected reflex increase in HR, which may reflect the ability of L-Wee to also exert a direct inhibitory effect on the heart.

While the endothelium-independent effect of L-Wee is less potent in aortic rings, the pattern of slow and gradual inhibition as well as the IC_{50} values we have noted in aortic rings are very similar to those reported previously using either a dipeptide (W-H) or a tripeptide (HRW). The addition of either L-W alone or L-W in combination with other amino acids as a mixture in the organ bath failed to evoke inhibition of KCl-evoked tension responses in aortic rings (Tanaka *et al.*, 2008; 2009; Wang *et al.*, 2010b). These data are consistent with our *in vivo* and as *in vitro* studies in which L-W *per se* failed to produce any inhibition. Based on blockade of KCl responses, Tanaka *et al.* have concluded that di- or tri-peptides containing L-W pass through the membranes and exert antagonism at the L-type VOCC by interacting at the phenylalkylamine (verapamil) binding site at the inner leaflet of the plasma membrane on VSMC of aorta. Clearly, more studies are warranted to resolve this issue. However, given the parallelism in the inhibition curves and strikingly similar IC_{50} values, it seems reasonable to suggest that the effects exerted by small peptides containing either L-Wee or L-W, on rat aortic rings are due to increased bioavailability of intracellular L-W, which then blocks the L-type VOCC. Alternatively, it is also possible that L-Wee but not L-W, interacts at an intracellular binding site (after gaining entry into VSMC) to exert L-type VOCC antagonism. In contrast, when L-Wee is rapidly degraded by tissue esterases to L-W soon after its entry into VSMC, its inhibitory effect is diminished. This may also account for the transient nature of the responses seen at lower concentrations and sustained inhibition encountered only at higher concentrations of L-Wee in MA rings. Similarly, the lower order of sensitivity and the slow pattern of inhibition of tension responses caused by either L-Wee or W-H/HRW may be related to their rapid breakdown by tissue esterases/peptidases to L-W, which does not interact at the intracellular binding site in conduit vessels such as the aorta. As the concentration of L-Wee is increased, our data suggests that a sufficient level of L-Wee is available in VSMC despite its potential breakdown to L-W to maintain sustained L-type VOCC antagonism. Clearly, more studies are warranted to address all these issues.

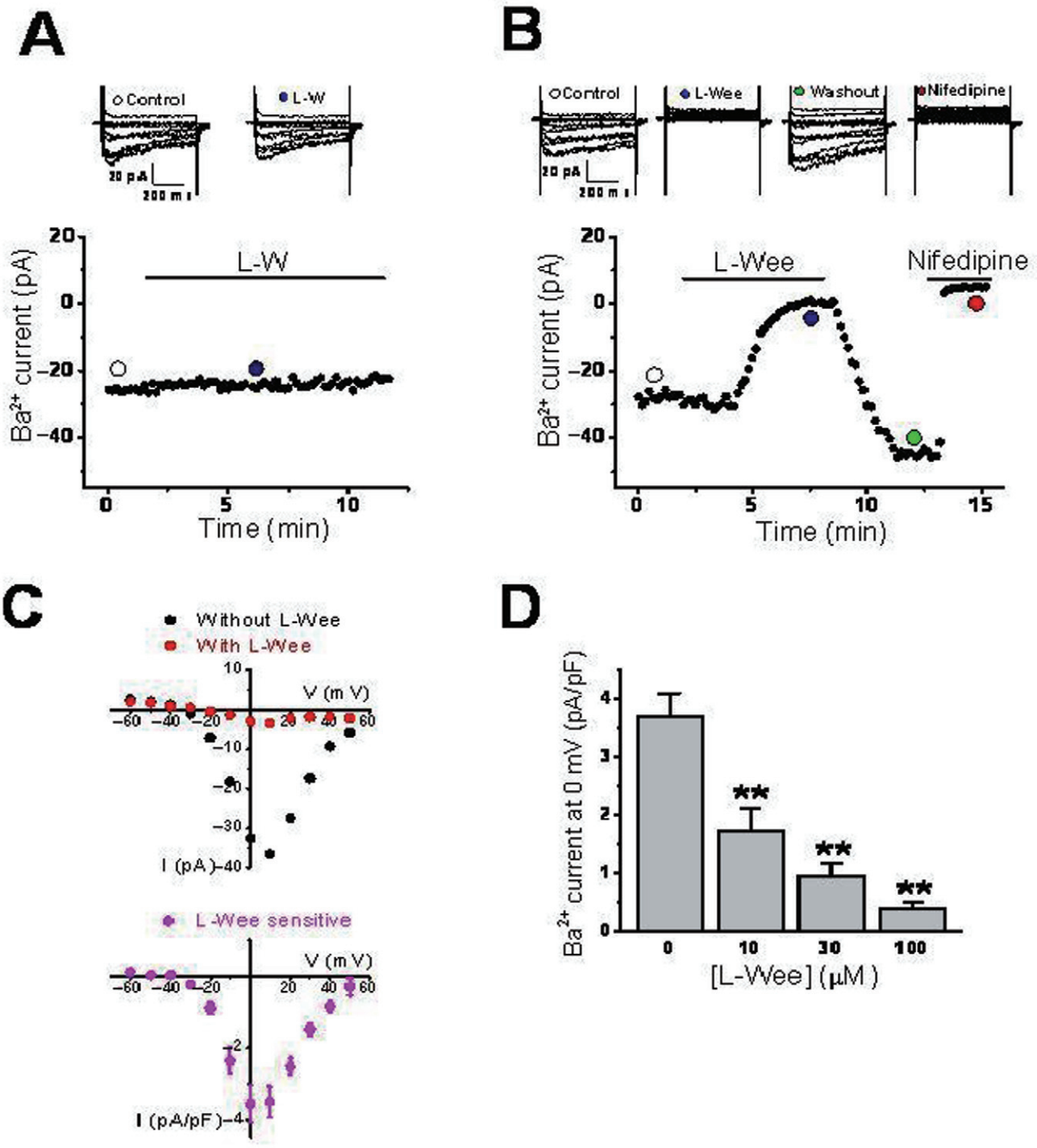

Figure 5

(A) Ba^{2+} currents recorded from a single VSMC before and after L-W (100 μM). Currents were elicited with voltage steps from –60 to +50 mV at time points as indicated in the time course (recorded at 0 mV) in lower panel. (B) Ba^{2+} currents recording before and after applications of L-Wee (100 μM) or nifedipine (5 μM). Lower panel shows the time course of currents at 0 mV. (C) Upper panel shows current–voltage relationships of nifedipine-sensitive currents in a VSMC before and after L-Wee (100 μM) application. Lower panel shows the current–voltage relationship of L-Wee sensitive currents (difference between currents before and after L-Wee treatment, $n = 6$). (D) Summary of mean ± SEM Ba^{2+} current density data in the presence of L-Wee at indicated concentrations ($n = 7$ VSMC from four rats). $^{**}P < 0.01$, significantly different from 0 μM.

The efficacy of the cell permeant L-Wee compared with the more polar L-W in blocking VOCC indicates that its mechanism of action is linked to its access to the VSMC intracellular compartment. In patch clamping experiments with isolated VSMC of MA, the time for the onset of VOCC inhibition was also found to be delayed (~90 s) compared with the rapid onset exerted by nifedipine (10–20 s), consistent with the increased time needed for L-Wee uptake into the cytosol. Moreover, the IC_{50} values for inhibition of L-type VOCC currents in isolated single VSMSC of MA and the inhibition of agonist-evoked vasoconstrictor responses in intact MA were also similar.

A recent study has shown that agents such as cinacalcet and calindol that are considered as activators of Ca^{2+} sensing receptors on the endothelium and thought to promote endothelium-dependent vasodilatation have turned out to be potent arterial vasodilators of MA via inhibition of L-type VOCC (Thakore and Ho, 2011). These authors have shown that these two agents act as Ca^{2+} channel antagonists (in low μM ranges) aas seen with L-Wee in MA. Whether these agents could affect VOCC in conduit and resistance-type vessels with differing sensitivity and also promote a fall in MAP and HR remains to be explored.

IN conclusion, L-Wee promoted significant degree of vasodilatation in small MA by blocking VOCC on VSMC, at plasma concentration ranges of L-W (Knott and Curzon, 1972). Another report suggests that tryptophan analogues could reduce vascular inflammation by reducing myeloperoxidase activity (Sliskovic *et al.*, 2009). Thus, the present study provides impetus to explore the potential role of several L-W ester analogues and indole group-containing drugs in blocking L-type VOCC on heart and different regions of the vasculature in the future.

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Conflict of interest

None.

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