Divergent signaling mechanisms for venous versus arterial contraction as revealed by endothelin-1

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Objective: **Venous function is underappreciated in its role in blood pressure determination, a physiologic parameter normally ascribed to changes in arterial function. Significant evidence points to the hormone endothelin-1 (ET-1) as being important to venous contributions to blood pressure. We hypothesized that the artery and vein should similarly depend on the signaling pathways stimulated by ET-1, specifically phospholipase C (PLC) activation. This produces two functional arms of signaling: diacylglycerol (DAG; protein kinase C [PKC] activation) and inositol trisphosphate (IP_3) production (intracellular calcium release).**

Methods: **The model was the male Sprague-Dawley rat. Isolated tissue baths were used to measure isometric contraction. Western blot and immunocytochemical analyses measured the magnitude of expression and site of expression, respectively, of IP_3 receptors in smooth muscle/tissue. Pharmacologic methods were used to modify PLC activity and signaling elements downstream of PLC (IP_3 receptors, PKC).**

Results: **ET-1-induced contraction was PLC dependent in both tissues as the PLC inhibitor U-73122 significantly reduced contraction in aorta (86% ± 4% of control; $P < .05$) and vena cava (49% ± 11% of control; $P < .05$). However, ET-1-induced contraction was not significantly inhibited by the IP_3 receptor inhibitor 2-aminoethoxydiphenylborane (100 μM) in vena cava (82% ± 8% of control; $P = .23$) but was in the aorta (55% ± 4% of control; $P < .05$). All three IP_3 receptor isoforms were located in venous smooth muscle. IP_3 receptors were functional in both tissues as the novel membrane-permeable IP_3 analogue (Bt-IP_3; 10 μM) contracted aorta and vena cava. Similarly, whereas the PKC inhibitor chelerythrine (10 μM) attenuated ET-1-induced contraction in vena cava and aorta (5% ± 2% and 50% ± 5% of control, respectively; $P < .05$), only the vena cava contracted to the DAG analogue 1-oleoyl-2-acetyl-*sn*-glycerol.**

Conclusions: **These findings suggest that ET-1 activates PLC in aorta and vena cava, but vena cava contraction to ET-1 may be largely IP_3 independent. Rather, DAG—not IP_3—may contribute to contraction to ET-1 in vena cava, in part by activation of PKC. These studies outline a fundamental difference between venous and arterial smooth muscle and further reinforce a heterogeneity of vascular smooth muscle function that could be taken advantage of for therapeutic development. (J Vasc Surg 2015;62:721-33.)**

Clinical Relevance: **These studies outline a new and fundamental difference between venous and arterial smooth muscle, in terms of excitation-contraction coupling and calcium mobilization during endothelin-1-induced contraction, and further reinforce the heterogeneity of vascular smooth muscle. Because changes in venous capacitance are associated with a multitude of medical conditions, including syncope, hemorrhage, shock, heat stroke, and congestive heart failure, these findings also present new potential therapeutic targets (specifically diacylglycerol interference) specific to veins.**

More attention has been given to the physiology of veins since researchers linked changes in venous capacitance to increases in blood pressure.[1] The role of veins in regulating blood pressure is still largely overlooked, even though it was noted more than 25 years ago that human hypertensive patients demonstrated impaired venous distensibility and decreased venous capacitance.[2,3] This change in distensibility could ultimately increase blood pressure by increasing arterial blood volume as the storage capacity of veins decreases. Nonetheless, the physiologic and signaling mechanisms regulating venous contraction are largely unexplored. They are assumed to be similar to those of arteries, but this assumption should be tested; this is the goal of this study. We test the general hypothesis that contraction to a hormone important to both arterial and venous function uses similar signaling pathways.

We focus on the functions of the hormone endothelin-1 (ET-1) because of the high potency of ET-1 in contracting venous tissue[4-6] and strong evidence

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that ET-1 supports venous function as a means to elevate blood pressure.[1,7,8] ET-1 is a 21-amino acid peptide, originally characterized as an endothelium-derived constricting factor in the vasculature.[9] The physiologic responses elicited by ET-1 are attributed to the two G protein-coupled receptors to which ET-1 binds: the ET_A and ET_B receptors.[10,11] As with other $G\alpha_q$-coupled receptors, both ET receptors can mediate Ca^{2+} release from intracellular stores by activation of phospholipase C (PLC) and the subsequent production of inositol trisphosphate (IP_3) and diacylglycerol (DAG).[12,13] Whereas sarcoplasmic reticulum Ca^{2+} release by ET-1 is primarily IP_3 mediated in arterial smooth muscle, the activation of protein kinase C (PKC) by DAG can inhibit IP_3 production to reduce IP_3-dependent Ca^{2+} release and contraction.[14,15] However, independent of PKC activation, DAG can directly activate several types of nonselective cation channels to increase Ca^{2+} influx and augment contraction.[16] This is true in rabbit portal vein, in which activation of PKC by DAG is necessary for IP_3-mediated Ca^{2+} signaling to occur.[17] This suggests that the mechanisms regulating Ca^{2+} release and $G\alpha_q$-mediated contraction vary widely, and the mechanisms are not wholly identical between arteries and veins.

This study tests the hypothesis that ET-1-mediated venous contraction, like arterial contraction, depends on PLC activation and both arms of PLC signaling—DAG and IP_3 production. Using pharmacologic inhibitors, we first investigated the role of PLC in ET-1-induced contraction in aorta and vena cava. We then examined how IP_3 and DAG can mediate contraction in aorta and vena cava and how contraction to ET-1 is mediated by IP_3 and DAG. Our results suggest that ET-1-induced contraction of rat aorta is mediated in part by IP_3, but ET-1-induced contraction in rat vena cava primarily involves DAG and is not likely to involve IP_3.

METHODS

Animal care and use. All procedures that involved animals were performed in accordance with the Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* at Michigan State University. Male Sprague-Dawley rats (250-300 g; 8-12 weeks old) were used. Animals were euthanized with sodium pentobarbital (60 mg/kg intraperitoneally).

Isometric contraction and compound source. Aorta and vena cava were dissected and cleaned of outer adipose tissue in physiologic salt solution containing the following (mM): NaCl, 130; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$, 1.17; $NaHCO_3$, 14.8; dextrose, 5.5; $Na_2EDTA \cdot 2H_2O$, 0.03; and $CaCl_2$, 1.6 (pH = 7.2). Endothelium-intact tissue rings were then mounted in warmed, aerated physiologic salt solution (37°C; 95%/5% O_2/CO_2) in isolated tissue baths (20 mL) for measurement of isometric contractile force with use of a 750TOBS Tissue Organ Bath System (Danish Myo Technology, Aarhus, Denmark) and PowerLab for Windows (ADInstruments, Colorado Springs, Colo). The tissues were placed under optimum resting tension (1 *g* for vena cava, 4 *g* for aorta)[11,18] and initially challenged with 10 μM norepinephrine (vena cava) or phenylephrine (aorta) to test for tissue viability. Different agonists were used for the initial challenge because vena cava does not respond to phenylephrine and to remain consistent with previously published work.[5,6,18,19] Endothelium viability was confirmed by relaxation to 1 μM acetylcholine after contraction by phenylephrine (aorta) or norepinephrine (vena cava). Tissues were washed every 15 minutes until they returned to resting tension. Cumulative concentration response curves or responses to single concentrations of agonists were recorded. Antagonists, inhibitors, or their vehicles were incubated with the tissues for 1 hour before addition of agonists. The specific agonists and antagonists, and corresponding solvents, were as follows: 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), acetonitrile/ethanol; 2-aminoethoxydiphenylborane (2-APB), dimethyl sulfoxide (DMSO); Bt-IP_3, DMSO; chelerythrine, DMSO; ET-1, dH_2O; norepinephrine, dH_2O; phenylephrine, dH_2O; U-37122, DMSO; and U-73343, DMSO. All compounds were purchased from Sigma-Aldrich Corporation (St Louis, Mo), with the following exceptions: Bt-IP_3 (SiChem, Bremen, Germany), ET-1 (1-21) (Bachem, Torrance, Calif), and OAG (Cayman Chemical, Ann Arbor, Mich).

Protein isolation and Western blot analysis. Endothelium-intact tissues were ground with mortar and pestle under liquid nitrogen in 1 mL of ice-cold homogenation buffer (50 mM Tris [pH 7.4], 4% sodium dodecyl sulfate, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin). The homogenate was vortexed, sonicated, transferred to a plastic centrifuge tube, and spun at 4°C to pellet debris; the supernatant was then kept. A bicinchoninic acid assay was used to determine protein concentration. Because of the high molecular weight of IP_3 receptor (IP_3R) protein (~300 kDa), Western blotting was performed with techniques for high-molecular-weight proteins as outlined in the current literature.[20-22] Samples (4:1 in denaturing sample buffer, boiled for 5 minutes) were separated on gradient (8%-15%) of Tris-acetate gels. Proteins were then wet transferred to nitrocellulose membrane at 30 V for 1 hour at 4°C. Membranes were blocked for 3 to 4 hours (phosphate-buffered saline [PBS], 5% Bio-Rad milk). Blots were probed for between 1 hour and overnight with primary antibody (rocking, at 4°C) and rinsed three times in PBS plus Tween (0.1%) with a final rinse in PBS and incubated with the appropriate secondary antibody for 1 hour at 4°C (rocking). Primary antibodies used included the following: anti-IP_3R-1 (1:1000; NeuroMab, Davis, Calif); anti-IP_3R-2 (1:1000; Millipore, Billerica, Md); and anti-IP_3R-3 (1:1000; Millipore). Methods of detection included standard ECL capture on film or digital capture by a LI-COR Fc (LI-COR, Lincoln, Neb). Band density was quantified with ImageJ software (National Institutes of Health, Bethesda, Md).

Smooth muscle cell dissociation and immunofluorescence. Whole aorta and vena cava tissues were isolated, cleaned of perivascular fat, and cut into ~1-mm rings.