Urotensin-II Signaling Mechanism in Rat Coronary Artery: Role of STIM1 and Orai1-Dependent Store Operated Calcium Influx in Vasoconstriction

Alejandro Domínguez-Rodríguez, Ignacio Díaz, María Rodríguez-Moyano, Eva Calderón-Sánchez, Juan Antonio Rosado, Antonio Ordóñez, Tarik Smani

Objective—Human urotensin-II (UII) is considered the most potent endogenous vasoconstrictor discovered to date, although the precise mechanism activated downstream of its receptor UTS2R in blood vessels remains elusive. The aim of this study was to determine the role of the store operated Ca2+ entry (SOCE) signaling pathway in UII-induced coronary artery vasoconstriction.

Methods and Results—We used a combination of isometric tension measurement, Ca2+ imaging, pharmacology, and molecular approaches to study UII-mediated rat coronary artery vasoconstriction and intracellular Ca2+ mobilization in coronary smooth muscle cells. We found that UII promoted dose-dependent vasoconstriction and elicited Ca2+ and Mn2+ influx, which were sensitive to classical SOCE inhibitors. In addition, knockdown of either STIM1 or Orai1 essentially inhibited UII-mediated SOCE and prevented UII but not high-KCL evoked contraction in transfected coronary artery. Moreover, we found that Ca2+-independent phospholipase A2 was involved in UII effects and that is colocalized with STIM1 in different submembrane compartments. Importantly, STIM1 but not Orai1 downregulation inhibits significantly independent phospholipase A2 activation. Furthermore, lysophosphatidylcholine, an independent phospholipase A2 product, activated Orai1 but not STIM1-dependent contraction and SOCE.

Conclusion—Here, we demonstrated that different critical players of SOCE signaling pathway are required for UII-induced vasoconstriction of rat coronary artery. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: calcium ■ coronary circulation ■ STIM1, Orai1, iPLA2

Urotensin II (UII) is a cyclic undecapeptide that is considered the most potent endogenous vasoconstrictor discovered to date in different mammals.1-2 In humans, resistance coronary arteries are extremely sensitive to UII compared to epicardial conduit arteries, implying a more important role for these resistance coronary arteries in regulating blood flow to the heart.3 UII binds to a Gq-protein coupled receptor, known as orphan receptor GPR14 or urotensin receptor (UTS2R).4-5 UTS2R activation results in the stimulation of phospholipase C (PLC) and inositol triphosphate (IP3) production, which triggers the release of Ca2+ from the sarcoplasmic reticulum (SR).6 UII and UTS2R are highly expressed within the human cardiovasculature (vascular SMC, endothelium, myocardium, etc.)7-8; and their overexpression have been implicated in a number of cardiovascular diseases, such as arteriosclerosis, heart failure, and hypertension.9,10 In rat aorta, UII increases the vascular tone, which was partially inhibited, although not abolished, by the L-type voltage-dependent Ca2+ channels (LTCC) antagonist verapamil.11 Other reports have suggested a role for RhoA and Rho-kinase, or for PKC and tyrosine kinase signaling pathways in UII-induced rat aorta contraction.12,13 However, the signaling pathway of UII is not fully established, especially in small resistance vessels such as coronary arteries.

It is now agreed that agonist-induced vascular SMC contraction is mediated by a rapid Ca2+ release by IP3 from intracellular stores and a transmembrane Ca2+ influx through LTCC and/or nonvoltage-gated channels such as store-operated Ca2+ channels (SOCC).14,15 Recently, we have demonstrated the importance of the store operated Ca2+ entry (SOCE) in agonist induced coronary artery vasoconstriction, and we have confirmed the critical role of Ca2+-independent phospholipase A2 (iPLA2) and its product lysophosphatidylcholine (LPC) in SOCE regulation and agonist evoked contraction.16 The discovery of STIM1 as the dynamic sensor...
of Ca\textsuperscript{2+} within the endo-sarcoplasmic reticulum (ER/SR), and Orai1 as the pore forming subunit of SOCC, have provided important information into the possible mechanism of SOCE.\textsuperscript{17,18} Decreased luminal Ca\textsuperscript{2+} activates STIM1 causing their aggregation and translocation to the plasma membrane where STIM1 couples directly to Orai1 allowing Ca\textsuperscript{2+} entry.\textsuperscript{19}

In the present study, we aimed to give further insights into the signaling pathway that mediates the effect of UII in coronary artery with special emphasis on SOCE regulated by STIM1, Orai1, and iPLA\textsubscript{2}.

**Methods**

An expanded Materials and Methods section detailing the protocols and techniques used in this study can be found in the Online Data Supplement, available online at http://atvb.ahajournals.org.

**Cells and Artery Preparation**

Isolated rat coronary arteries were dissected from the heart of adult male Wistar rats weighing 250 to 450 g and cells culture were prepared as previously described.\textsuperscript{16} Isometric tension was measured in rat coronary arterial rings (~2 mm) using a small-vessel myograph (JP Trading,.auhhus, Denmark) connected to a digital recorder (Myodataq-2.01, Myodata-2.02 Multi-Myograph System) as previously described.\textsuperscript{16}

**Intracellular Ca\textsuperscript{2+} Measurement**

Ca\textsuperscript{2+} and Mn\textsuperscript{2+} measurement were carried out in isolated SMC loaded with 2 to 5 \( \mu \)mol/L Fura-2 AM using an imaging system (InCyt Im2, Intracellular Imaging, Insol, UK) equipped with a lightsensitive CCD camera (Cooke PixeFly, ASI, Eugene, OR) as described previously.\textsuperscript{16}

**Results**

**UII Mediates Vasocostriction Through UII Receptors, UTS2R, in Coronary Artery**

First, we examined the effect of UII on coronary artery contraction. Figure 1A shows that the cumulative application of UII produced a concentration-dependent vasoconstriction in endothelium-denuded coronary arterial rings. Maximal contraction was observed when UII was applied at 100 \( \mu \)mol/L concentration and the \( EC_{50} = 21.2 \pm 1.3 \) \( \mu \)mol/L comparing to the vasoconstriction induced by 60 mmol/L KCL. To demonstrate the involvement of UTS2R in UII effects in coronary artery, we first confirmed by Western blot the presence of UTS2R in both rat coronary artery and in primary culture of coronary SMC (Figure 1B insert). Next, we examined the effect of urantide, suggested as the most powerful peptide based antagonist of UTS2R,\textsuperscript{22} on vessels contraction and Ca\textsuperscript{2+} mobilization. As illustrated in Figure 1B, urantide at 100 \( \mu \)mol/L significantly prevented UII (20 \( \mu \)mol/L)-induced contraction, whereas the response to high-KCL was unaffected. Furthermore, the addition of UII (20–50 \( \mu \)mol/L) in isolated coronary SMC evoked an intracellular Ca\textsuperscript{2+} increase that was significantly inhibited by urantide (Figure 1C). UTS2R is functionally linked to Gq11 protein whose activation stimulates PLC, hence the PLC inhibitor U73122 was tested. Figure 1C shows that UII-induced intracellular Ca\textsuperscript{2+} mobilization was significantly blocked in cells pretreated 10 minutes with U73122 (50 \( \mu \)mol/L). Moreover, none of the rings tested in the presence of U73122 showed any significant response to 100 nmol/L UII (data not shown). These data confirm that UII stimulates UTS2R and PLC cascade to induce intracellular Ca\textsuperscript{2+} increase and coronary vasoconstriction.

**Role of SOCE in UII Induced Vasocostriction**

Next, we determined whether UII activation of PLC/IP\textsubscript{3} signaling cascade would stimulate a functional SOCE pathway in short-term cultured coronary SMC. Figure 2A and 2C shows that UII at 50 nmol/L applied in the absence of extracellular Ca\textsuperscript{2+} induced transient cytosolic Ca\textsuperscript{2+} release from SR. The readdition of extracellular Ca\textsuperscript{2+} evoked Ca\textsuperscript{2+} influx similar to that typically evoked by passive depletion of SR with TG (2 \( \mu \)mol/L, Figure 2B and 2C). SMC treatment with pharmacological inhibitors of SOCE,\textsuperscript{23} Gd\textsuperscript{3+} (5 \( \mu \)mol/L), 2APB (50 \( \mu \)mol/L), and DES (1 \( \mu \)mol/L)\textsuperscript{24} significantly blocked UII and TG-evoked Ca\textsuperscript{2+} influx in coronary SMC. On the other hand, we used the Mn\textsuperscript{2+}-quench technique to confirm SOCE activation by UII and to exclude the influence of mechanisms that remove Ca\textsuperscript{2+} from the cytoplasm.\textsuperscript{23,25} Figure 2D–2F shows that UII induced an influx of Mn\textsuperscript{2+} alike that induced by TG, which was also sensitive to 2APB (50 \( \mu \)mol/L), DES (1 \( \mu \)mol/L), and Gd\textsuperscript{3+} (5 \( \mu \)mol/L).

We next investigated SOCC implication in UII-evoked coronary vasoconstriction. Figure 3A shows that UII (20 nmol/L) applied in Ca\textsuperscript{2+} free solution elicited a transient contraction, and the subsequent addition of Ca\textsuperscript{2+} (2 mmol/L) in presence of nifedipine (0.5 \( \mu \)mol/L) led to a sustained contraction that was significantly antagonized either by 2APB (50 \( \mu \)mol/L) or by Gd\textsuperscript{3+} (5 \( \mu \)mol/L, mean data in Figure 3A). Additionally, the contraction induced by Ca\textsuperscript{2+} restoration in the absence of nifedipine was significantly higher than in rings treated with nifedipine (mean data in Figure 3A). Moreover, as illustrated in Figure 3B–3E, the addition of UII in normal Krebs solution (containing 2 mmol/L Ca\textsuperscript{2+}) evoked contraction that was effectively relaxed with Gd\textsuperscript{3+} (5–10 \( \mu \)mol/L), 2APB (50 \( \mu \)mol/L), or DES (1 \( \mu \)mol/L).

**STIM1 and Orai1 Involvement in UII Evoked Ca\textsuperscript{2+} Entry and Contraction**

Once we determined the role of SOCE in UII-mediated contraction, we investigated the participation of STIM1 and Orai1 in UII effects. First, we verified that RNA knockdown of STIM1 and Orai1 specifically reduced the expression level of their mRNA in coronary SMC (Supplemental Figure Ia, available online at http://atvb.ahajournals.org), and their target proteins as assessed by western blotting (Figure 4A upper panel and Supplemental Figure Ib). Importantly, STIM1 and Orai1 disruption significantly suppressed UII-elicited Ca\textsuperscript{2+} (Figure 4A) and Mn\textsuperscript{2+} influx (Figure 4B). In similar conditions, Ca\textsuperscript{2+} entry mediated by passive store depletion with TG was abolished in cells transfected with either STIM1 or Orai1 siRNA (Supplemental Figure Ic).

Furthermore, to assess the role of STIM1 and Orai1 as determinant proteins responsible for UII-activated Ca\textsuperscript{2+} entry and the consequent vasoconstriction, we examined UII application in coronary arterial rings transfected with STIM1 or
Figure 1. Urotensin-II (UII) induces potent vasoconstriction and calcium mobilization through UTS2R. 

**A**, Representative recording of UII evoked concentration-dependent contraction in endothelium-denuded rat coronary artery. Right panel shows the average of concentration-response for UII-induced contraction normalized to high-KCL (60 mmol/L) response. The fit was generated with Hill equation ($EC_{50}$=21.2±1.3 nmol/L, n=7). B, The insert shows by Western blot the presence of UTS2R in primary cultured coronary smooth muscle cells (SMC) and in coronary artery (cor. artery). Lower panel shows 2 independent rings and summary data (right panel), treated with Urotensin-II (UII, 20 nmol/L) and high-KCL (60 mmol/L) in presence (gray line) or not (black line) of urantide (100 nmol/L). Values are mean±SEM tension (% of KCL response, n=6). C, Representative traces and summary data of UII induced intracellular Ca$^{2+}$ mobilization in isolated SMC loaded with fura 2AM. UII (50 nmol/L) was added to standard solution in presence of Ca$^{2+}$ in control cells, in cells pretreated 10 minutes with Urantide (100 nmol/L), or with PLC inhibitor (U73122, 50 μmol/L). The summary data correspond to large number of cells from 3 primary cultures. *P<0.01.
Orai1 siRNA. STIM1 and Orai1 downregulation was effective in transfected rings (Figure 4C and Supplemental Figure IIa, available online at http://atvb.ahajournals.org) which inhibited UII-induced contraction, meanwhile high-KCl-activated contractions were not affected comparing to the response in rings transfected with scramble RNA (Figure 4C). Consistently, Orai1 and STIM1 downregulation also inhibited phenylephrine evoked contraction (Supplemental Figure II).

iPLA2 Involvement in UII Effects

Several reports have determined the crucial role of iPLA2 in SOCE (for review, see Ref. 26). Here, we explored the potential involvement of iPLA2 modulation of SOCE in UII actions. Figure 5A shows that SMC treatment with 50 nmol/L UII stimulated iPLA2 activity; which was abolished in cells preincubated with urantide, UTS2R antagonist. In addition, SMC pretreatment with iPLA2 inhibitor bromoenol lactone (BEL, 25 μmol/L) significantly blocked iPLA2 activation by UII. Next, we compared the effects of S-BEL and R-BEL, chiral enantiomers of BEL capable to discriminate between iPLA2 isoforms β and γ, respectively.27,28 Figure 5A shows that UII-evoked iPLA2 activation was inhibited in SMC treated with S-BEL (specific to iPLA2β) but not with R-BEL (specific to iPLA2γ). Furthermore, UII-elicted Ca2+ (Figure 5B) and Mn2+ influx (Figure 5C) were prevented by BEL (25 μmol/L) and specifically inhibited by S-BEL (25 μmol/L) but not by R-BEL (25 μmol/L). Interestingly, UII evoked contraction was also significantly relaxed by BEL (25 μmol/L) and S-BEL (25 μmol/L), but not by R-BEL (25 μmol/L) as shown in Figure 5D–5F. These data confirm that
UII requires active iPLA2β to promote Ca^{2+} entrance and artery contraction.

Afterward, we performed several set of experiments to examine the role of iPLA2β stimulation in SMC and vessels lacking STIM1 and Orai1. Figure 6A shows that UII activation of iPLA2 was attenuated in SMC transfected with siRNA for STIM1 but not by Orai1 knockdown. Next, we used immunofluorescence to elucidate the relationship between endogenous STIM1 and iPLA2. Figure 6B and Supplemental Figure III and IV, available online at http://atvb.ahajournals.org, show that in resting cells STIM1 and iPLA2 are distributed uniformly through cells compartments. However, cell treatment with 50 nmol/L UII or with 2 μmol/L TG resulted in STIM1 redistribution in the subplasma membrane region that was clearly colocalized to iPLA2 as shown in the magnified picture and graph detail in online Figure IV. These data indicate that iPLA2 could colocalize with STIM1 and require at least in part STIM1 to promote UII action.

Orai1 but not STIM1 Downregulation Inhibited Lysophosphatidylcholine-Induced SOCE and Contractions

The activation of iPLA2 produce lysophospholipids that are known to activate SOCE. We tested whether LPC could mimic the effects of UII. First, LPC (300 nmol/L) activated 2APB and Gd^{3+} sensitive Ca^{2+} influx and coronary artery contraction as shown in Supplemental Figure V, available online at http://atvb.ahajournals.org. Considering that LPC is...
Figure 4. Knockdown of STIM1 or Orai1 prevents Urotensin-II (UII) evoked store operated Ca\(^{2+}\) entry in smooth muscle cells (SMC) and contraction in coronary artery. **A**, Upper panel, Western blot showing significant downregulation of STIM1 and Orai1 proteins in primary culture of coronary SMC, 72 hours after siRNA transfection. **A**, Lower panel, and **B** representative traces and summary data illustrating Ca\(^{2+}\) and Mn\(^{2+}\) influx in SMC treated with UII (50 nmol/L) for 3 to 4 minutes in the absence of extracellular Ca\(^{2+}\), and then 2 mmol/L Ca\(^{2+}\) (A) or 200 \(\mu\)mol/L Mn\(^{2+}\) (B) were added as indicated. Traces are for SMC transfected 72 hours prior to the experiments with scramble siRNA (scramble), STIM1 siRNA (siSTIM1), or Orai1 siRNA (siOrai1). Basal indicates Ca\(^{2+}\) or Mn\(^{2+}\) influx in untreated SMC; control, nontransfected cells treated with UII. \(n=\)cells from 4 to 7 different transfections. * and ** denote significance at \(P<0.01\) and \(P<0.05\), respectively, comparing to scramble. **C**, Upper panel, Western showing significant downregulation of STIM1 (49.9±4.4%, \(n=3\)) and Orai1 (55.4±9.4%, \(n=3\)) proteins in coronary artery, 48 hours after their transfection. Lower panel shows traces of vascular tone from three independent rings (left), and summary data (right), transfected with scramble siRNA (scramble), with siRNA for STIM1 (siSTIM1), and with siRNA for Orai1 (siOrai1). UII (50 nmol/L) was applied as indicated, then washed out for 30 minutes. High-KCL (60 mmol/L) was added as a control. Values normalized to KCL response are means±SEM from 5 to 8 different experiments. **P<0.05.
a ligand of certain Gq-protein coupled receptor in some cell line,29 we found that LPC (300 nmol/L) induced Ca$^{2+}$/H$^{+}$ influx was not affected by PLC inhibition with U73122 (50 nmol/L, Supplemental Figure VI, available online at http://atvb.ahajournals.org). Next, in SMC transfected with siRNA STIM1, LPC (300 nmol/L) stimulated practically the same Ca$^{2+}$/H$^{+}$ and Mn$^{2+}$/H$^{+}$ influx comparing to those transfected with scramble siRNA (Figure 6C and 6D). Interestingly, the disruption of STIM1 in small rings also failed to inhibit LPC-induced contraction as illustrated in Figure 6E. However, LPC mediated Ca$^{2+}$ and Mn$^{2+}$ entry in cells, or contractions in vessels transfected with Orai1 siRNA were significantly attenuated (Figure 6C–6E), confirming that LPC require Orai1 but not STIM1 to promote SOCE and contraction. Figure 6E also shows that LPC-evoked contractions in transfected rings were effectively antagonized by 2APB.

Figure 5. Urotensin-II (UII) activates independent phospholipase A$_2$β (iPLA$_2$β) that is involved in UII-evoked store operated Ca$^{2+}$ entry and vasoconstriction. A, Mean data of iPLA$_2$ activity (normalized to control) in smooth muscle cells (SMC) treated 3 to 4 minutes with UII (control, 50 nmol/L); in cells pretreated 5 minutes with urantide (+ urantide, 100 nmol/L) then 3 minutes with UII. +BEL, +S-BEL, and + R-BEL indicate cells incubated with 25 μmol/L of BEL, S-BEL, and R-BEL, respectively, 30 minutes at 37°C before treatments with Urotensin-II; basal, untreated SMC. Values are means ± SEM from 3 to 5 cultures of SMC. B, C, Show summary data of Ca$^{2+}$ and Mn$^{2+}$ influx after 2 mmol/L Ca$^{2+}$ or 200 μmol/L Mn$^{2+}$ addition in SMC treated with UII (control, 50 nmol/L). Data are also from cells pretreated with 25 μmol/L of BEL, S-BEL, and R-BEL (30 minutes at 37°C) before UII addition. Summary data are from 5 cultures. D, E, Show representative traces and (F) summary data of UII (20 nmol/L) induced contractions treated with 25 μmol/L of BEL; S-BEL, and R-BEL as indicated. n=6 to 10 rings. *$P<0.01$ treatments vs. UII; **$P<0.01$ S-BEL versus R-BEL.
Figure 6. STIM1 and independent phospholipase A$_2$, iPLA$_2$ (iPLA$_2$) colocalization; and requirement of Orai1 but not STIM1 for lysophosphatidylcholine-evoked store operated Ca$^{2+}$ entry and vasoconstriction. A, Bar graph showing the iPLA$_2$ activity (normalized to control) in smooth muscle cells (SMC) treated 3 minutes with Urotensin-II (UII; control, 50 nmol/L); from cells transfected with either scramble siRNA; STIM1 siRNA (siSTIM1); or Orai1 siRNA (siOrai1). Values are means±SEM (n=3 cultures), **P<0.05. B, Immunofluorescence staining with specific antibodies shows double localization of iPLA$_2$ (red) and STIM1 (green) in SMC stained with anti-rabbit iPLA$_2$, and anti-mouse STIM1 antibodies. Images are for untreated SMC (control) and for SMC treated with UII (50 nmol/L). UII (50 nmol/L) addition induces STIM1 redistribution in small punctae which show high colocalization with iPLA$_2$ as indicated by yellow color in merged magnified images. C, D, Representative traces and summary data illustrating Ca$^{2+}$ and Mn$^{2+}$ entry in SMC treated with lysophosphatidylcholine (LPC, 300 nmol/L) for 3 to 4 minutes in the absence of extracellular Ca$^{2+}$, and then 2 mmol/L Ca$^{2+}$ or...
These data indicate that STIM1 are upstream and Orai1 downstream from iPLA$_2$ stimulation and its product LPC which activates SOCE and contraction in coronary artery. Altogether, our study suggests that UII activation of UTS2R induces a potent vasoconstriction that involves STIM1, iPLA$_2$, and Orai1 dependent Ca$^{2+}$ entry.

Discussion

Several reports have agreed on the growing importance of UII action in a variety of diseases such as hypertension, arteriosclerosis, heart failure, diabetes, and so on.$^{30}$ Recently, we have shown that UTS2 gene associates with type 2 diabetes mellitus in European population.$^{31}$ UII physiological mechanism is still under debate even though it seems similar to other potent vasoactive agonists. It elicits a strong vasoconstriction via mobilization of intracellular Ca$^{2+}$ as well as through extracellular Ca$^{2+}$ entry.$^5$ This study presents the first evidences of the essential role of SOCE in UII-induced vasoconstriction. We have shown that (1) UII was effective at the nanomolar range confirming that it is among the most potent endogenous vasoconstrictors in rat coronary arteries consistent with its effects in other vessels.$^5$ (2) UII evoked typical SOCE and contractions that were pharmacologically inhibited with low concentration of Gd$^{3+}$, 2APB, and DES, the widely used inhibitors of SOCE in different cell types.$^{23}$ (3) Silencing of STIM1 or Orai1 drastically blocked UII-stimulated Ca$^{2+}$ and Mn$^{2+}$ entry and contraction. (4) iPLA$_2$, STIM1, and Orai1 was required for both UII-evoked Ca$^{2+}$ entry and contraction. (5) UII-mediated iPLA$_2$ activation required functional STIM1 that was localized in the proximity of iPLA$_2$. (6) LPC activated Orai1 but not STIM1 dependent SOCE and vasoconstriction.

We have shown that UII requires UTS2R and PLC signaling cascade, and that SOCC are involved in UII-induced coronary constriction, which indicates for the first time the important role of SOCC in UII signaling pathway, LTCC seems also involved in UII-evoked contraction although their inhibition only decreased partially UII response. Consistently, we have previously shown that SOCC activation involves LTCC stimulation in coronary artery.$^{16}$ Other reports have also suggested a role for LTCC, Rh0/A/Rho-kinase, PKC, and tyrosine kinase in UII-induced rat aorta contraction.$^{11-13}$

The new advances in SOCE field have identified STIM and Orai proteins as key molecular signaling players in a number of cell types and have determined their interaction to promote SOCE.$^{17-19}$ However, only few reports have studied STIM1- and Orai1-mediated SOCE in SMC other than cultured aortic SMC.$^{32,33}$ To our knowledge, the present study is the first to provide evidences for the existence of endogenous STIM1 and Orai1 proteins in rat coronary artery and SMC. We have showed that downregulation of STIM1 and Orai1 prevented UII-induced Ca$^{2+}$ and Mn$^{2+}$ entry in the same way as it inhibited SOCE induced by TG. Importantly, RNA silencing of STIM1 and Orai1 in transfected coronary small ring effectively attenuated UII-evoked contraction. In these conditions, similar effects were also observed when phenylephrine, which activates the G-protein/PLC cascade, was tested. Meanwhile, high-KCl stimulated contractions were not affected by Orai1 neither by STIM1 downregulation, indicating the specific effect of the siRNA and the adequate integrity and function of the artery after vessels transfection. A previous study has determined that antibodies against Orai1 and STIM1 partially prevented TG-induced aorta contraction$^{33}$; although our data are the first to functionally demonstrate that STIM1 and Orai1 are essential for agonist-induced artery contraction. Knockdown of Orai1 in SMC shows a residual Ca$^{2+}$ entry that could be due to the remaining expression of Orai1 and/or to the store-independent mechanisms such as TRPC channels activated downstream of PLC and positively regulated by IP$_3$.$^{34}$

STIM1 is regulating an increasing number of ion channels, such as Orai2 and Orai3,$^{35}$ LTCC,$^{36,37}$ several TRPC channels,$^{38}$ and even transporters.$^{39}$ Here, we showed that STIM1 downregulation attenuated iPLA$_2$ activation. Moreover, on cells stimulation STIM1 colocalized with iPLA$_2$ in submembrane compartments indicating that might communicate functionally. Multiple activation models for SOCE have been proposed and mechanisms linking iPLA$_2$ to SOCE are still under debate$^{26,40}$ although iPLA$_2$ seems essential for SOCE activation in SMC and a variety of arteries,$^{16,21,28}$ as well as in other excitable and nonexcitable cells as reviewed by Bolotina.$^{30}$ Here, we found that LPC-mediated SOCE and vasoconstriction were dependent on Orai1 but not on STIM1. These data suggest that STIM1 are upstream and Orai1 downstream, from iPLA$_2$ activation and its product LPC which might be required as functional transducers after agonist stimulation, from STIM1 in the SR to Orai1 in the plasma membrane. This hypothesis could be explained by the finding from Balla’s group who demonstrated the presence of additional molecular components within the STIM1-Orai1 complex.$^{41}$ Therefore, we believe that this finding is not incompatible with direct STIM1–Orai1 interactions and may point toward the involvement of auxiliary proteins that might coactivate or intermediate between STIM1–Orai1 interaction as discussed previously.$^{26,40}$

In summary, we have demonstrated that UII induces a potent vasoconstriction of rat coronary artery, which requires UTS2R/PLC signaling cascade, STIM1, iPLA$_2$, and Orai1 activation to enhance intracellular Ca$^{2+}$ and to evoke vessel contraction as proposed in a simplified model in Figure 7. Our results could have a wide functional relevance for both physiological and pathophysiological function of coronary artery SMC and their regulation by potent vasoconstrictors such as UII. Future SOCC antagonists would be beneficial in...
the therapeutic control of SMC function during different vascular pathologies.

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Disclosures
None.

References


29. Sáez ME, Smani T, Ramírez-Lorca R, Díaz I, Serrano-Rios M, Ruiz A, Ordóñez A. Association analysis of urotensin II Gene (UTS2) and


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METHODS

All the experiments were performed in accordance with the animal care guidelines of the European Communities Council (86/609/EEC), and this study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication N° 85-23, revised 1996). All procedures were approved by the Bioethical Committee of the Institute of Biomedicine of Seville (IBiS).

Cells And Arteries Preparation.

Adult male Wistar rats weighing 250–450 g were heparinized (4 IU/g i.p.) and anaesthetized by intraperitoneal administration of ketamine hydrochloric acid (1-2 ml/250 g). The hearts were quickly removed and placed in cold Krebs solution. Primary culture of coronary SMC was prepared following the same protocol as described previously. Isolated coronary arteries were dissected from the heart, cleaned and cut into pieces. They were incubated with 1 mg/ml elastase and 2 mg/ml collagenase in Dulbecco’s Modified Eagle’s Medium (DMEM, PAA labs.) for 30 min at 37°C. Cells were mechanically dispersed, plated on coverslips, and grown in DMEM supplemented with 10% fetal bovine serum (FBS, PAA labs.), with 100 U/ml penicillin and streptomycin in humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells exhibited positive fluorescence with antibodies against Û-smooth muscle actin.
Measurement Of Contractility In Arterial Rings.

Rat coronary arteries were obtained from Wistar male rats. Arteries were cleaned of connective tissue, cut in rings (∼2 mm) and mounted on a small-vessel myograph (JP Trading, Aarhus, Denmark) to measure isometric tension connected to a digital recorder (Myodataq-2.01, Myodata-2.02 Multi-Myograph System) as previously described. The rings were placed on a chamber filled with the physiological salt solution (PSS) and bubbled with 95% O₂ and 5% CO₂. Before the experiments, the segments were subjected to an optimal tension (90% of the vessels diameter when relaxed and under an intramural pressure of 100 mmHg) and stabilized for at least 1 hour following Mulvany’s standard methods. The endothelium was mechanically removed by rubbing the luminal surface of the ring with a small plastic tube. The functional removal or the integrity of the endothelium was tested in the beginning of each experiment by the addition of adenosine diphosphate (ADP), an endothelium dependent inductor of nitric oxide production.

Summary data presented in bar graphs show the increment (difference between maximum contraction and resting tone) of the vasoconstriction normalized to high-KCL (60 mM) induced contraction. Experiments were performed at 37°C. The composition of PSS was (in mM): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 5 glucose.

Ca²⁺ Measurement And Mn²⁺ Influx.

SMC plated on 30 mm round coverslips and mounted in a Teflon chamber were incubated in DMEM with 2-5 µM fura-2 AM for 30 minutes at 37°C then the cells were washed. Fluorescence was monitored using a Nikon TS-100 inverted microscope equipped with a 20X fluor objective (0.75 NA) as described previously. Fluorescence images of 20 to 30 cells were recorded and analysed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Imsol, UK) equipped with a light-sensitive CCD camera (Cooke PixelFly, ASI, Eugene, OR, USA). Changes in intracellular Ca²⁺ are represented as the ratio of fura-2 fluorescence induced at an emission wavelength of 510 nm due to excitation at 340 nm and 380 nm (ratio = F₃₄₀/F₃₈₀). Experiments were done in free Ca²⁺ solution (in mM: 140 NaCl, 2.7 KCl, 4 MgCl₂, 0.5 EGTA, 10 hepes), and the Ca²⁺ influx was determined from changes in fura-2 fluorescence after re-addition of
Ca\textsuperscript{2+} (2 mM). Ca\textsuperscript{2+} influx (Δratio) was calculated as the difference between the peak ratio after extracellular Ca\textsuperscript{2+} was added and its level right before Ca\textsuperscript{2+} addition.

The rate of Mn\textsuperscript{2+} influx-induced fura-2 quenching was used to estimate SOCE into SMC as we described previously.\textsuperscript{1,4} Fura-2 was measured in Ca\textsuperscript{2+} free solution at 360 nm, and the fluorescence was normalized to the value measured immediately before the addition of 200 µM Mn\textsuperscript{2+}. The rate of influx was estimated from the slope during the first 20 seconds after Mn\textsuperscript{2+} addition.

**SMC Protein Extract Preparation.**

Coronary SMC were seeded in 60 mm petri-dishes and cultured until confluence. After each treatment SMC were collected, homogenized in RIPA buffer (50 mM Tris-HCl, ph 8, 150 mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS and 0.2 mM EDTA) supplemented with 1mM PMSF and 10% cocktail of protease inhibitors (Sigma-Aldrich), sonicated and centrifuged at 20,000g for 15 min at 4°C. The supernatant was collected and the amount of protein was determined using the Bio-Rad protein dye reagent (Bradford method). The samples were aliquoted (30 µg each), frozen and stored at -80°C until later use.

**iPLA\textsubscript{2} Activity.**

iPLA\textsubscript{2} activity was performed using a modified kit from Cayman Chemical as described previously.\textsuperscript{1,3} iPLA\textsubscript{2} activity was assayed by incubating the samples with the substrate, arachidonoyl thio-PC, for 1 hour at room temperature in a modified Ca\textsuperscript{2+}-free assay buffer of the following composition (in mM): 300 NaCl, 10 HEPES, 8 Triton X-100, 4 EGTA, 60% glycerol, and 2 mg/ml of BSA (pH 7.4) as described.\textsuperscript{17,26} The generated free thiols were visualized by the addition of DTNB for 5 min, and the absorbance was determined at 405 nm using a standard microplate reader. The specific activity of iPLA\textsubscript{2} was expressed in absorbance/µg protein units and was normalized to control values.

**RNA Extraction and Quantitative RT-PCR.**

Total RNA was extracted from cultured SMC using a Qiagen Rneasy mini Kit following the manufacturer's protocol. cDNA was reverse transcribed using oligo(dT) 20 primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) from 1 µg of RNA. Real-time PCR analysis for
Orai1, STIM1, and beta-actin were performed using predesigned Taqman Gene Expression assays from Applied Biosystems, including primers and probes. Quantitative PCR amplification was performed using a Stratagene MX3005P thermal cycler with the following conditions: 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 30 seconds and 60ºC for 1 minute. Quantification was measured as sample fluorescence crossed a predetermined threshold value that was just above the background. Orai1 and STIM1 expression values were calculated using the comparative \( \Delta \Delta Ct \) method, where beta-actin was the housekeeping gene as described previously.\(^1\)

**Western Blotting.**

Protein samples were extracted from coronary arteries in RIPA buffer using a mortar and pestle and cultured cell lysates from coronary SMC were obtained in RIPA using a scraper. Equal amount of protein were subjected to SDS-PAGE (10%) and electrotransferred onto PVDF membranes. After blocking with 5% non fat dry milk dissolved in Tris-buffered saline containing 0,1% Tween-20 (TTBS) for 2 hours at room temperature, western blots were probed overnight at 4ºC with specific primary antibodies in TTBS containing 5% BSA. After washing, membranes were incubated for 45 minutes at room temperature with a horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Jackson IR.) in TTBS. Detection was performed with the enhanced chemiluminescence reagent ECL-plus (Amersham Bioscience). Primary antibodies used were: Orai1 (D90B10) rabbit Ab (1:1000 dilution), STIM1 rabbit Ab (1:1000 dilution) from Cell Signaling, anti-Urotensin II receptor U5508 (1:500 dilution), monoclonal anti-β-Actin clone AC-15 (1:5000) from Sigma-Aldrich, and anti β-Tubulin ab6046 (1:1000) from Abcam. For quantification, films were scanned and tiff images were analysed with Image J software using β-Actin or β-Tubulin as housekeeping loading control.

**Cell Transfections And Gene Silencing.**

Coronary SMC were seeded on 6-well plates and cultured until they reached 70-80% confluence. Predesigned small interference RNA (siRNA) sequences to Orai1, STIM1 and scramble as negative control were from Ambion. Transfection of siRNAs molecules was performed using HiPerFect transfection reagent (Qiagen). 10 nM of oligos were mixed with 12 µl Hiperfect reagent in 100 µl of OptiMem (Invitrogen) and used to transfect in 900 µl OptiMem per well. After 3
hours, 2 ml of complete medium were added and cells were cultured in normal conditions during 72 hours until Ca$^{2+}$ experiments, RNA or protein extraction. Information related to siRNA sequences and Taqman gene expression assays are shown in table 1 and table 2 respectively.

**Artery Transfection By The Chariot Technique.**

siRNA molecules were intracellularly delivered by the Chariot technique (Active Motif). This transfection reagent is able to deliver antibodies and other smaller molecules into cells. Coronary rings were incubated in DMEM for 30 minutes at 37ºC. For each transfection, 10 μl of Chariot in 100 μl distilled water were mixed with 3 μl siRNA molecules (10 nM final) in 100 μl of PBS and incubated at room temperature for 30 minutes to allow the complex to form. Coronary artery was transferred to a sterile 24-well cell culture plate, overlaid with 200 μl of Chariot complex and mixed gently. 400 μl Optimem was added and the tissues were incubated for 1 hour at 37ºC then tissues were supplemented by 750 μl of DMEM and let to incubate for 48 hours at 37º C.

**Immunofluorescence.**

Cells were grown in coverslips and acid washed in HCl. After treatments cells were fixed with 4% (w/v) fresh paraformaldehyde for 10 minutes in PBS, treated for 1 hour with 3% heat inactivated goat serum, 1% BSA, 0.1% Triton X-100 in PBS (for blocking) and incubated in a humidified chamber overnight at 4ºC with the primary antibodies solution (Rabbit anti-iPLA$_2$ Cayman Chemical 1:100 dilution and mouse anti-STIM1 BD Biosciences 1:50 dilution). After PBS washes, coverslips were incubated for 1 hour in dark with the secondary antibodies solution (Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse Molecular Probes 1:100 dilution). Coverslips were mounted with Vectashield medium with DAPI (Vector Labs) and fluorescent signal was visualized on Leica TCS SP2 confocal microscopy. Images were acquired of one focal plane with HCX PL APO 63x Glyc objective. Measurement analysis was done by linear profile tool, where it represents the channels intensity of a linear tracing on graphic. As a negative control, cells were incubated with secondary antibodies only.

**Drugs and Statistical Analysis.**

Human Urotensin-II is a synthetic peptide purchased from Sigma. The rest of drugs were from Sigma-Aldrich, Cayman Chemical, Promega and Invitrogen. Group data are presented as
mean ± S.E.M. Single or paired Student’s t test was used to determine the statistical significance of the obtained data. Significance between multiple groups was evaluated using ANOVA followed by Tukey test.

**References**


**Table 1. Small interference RNA sequences**

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Table 2. Taqman gene expression assays

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Online Figure Legends

Online figure I.

* a) Quantitative PCR shows that siRNA for STIM1 (left panel) and Orai1 (right panel) specifically and efficiently reduced their target mRNA, 72 hours after SMC transfection. Bar graphs show the relative fold expression level of STIM1 and Orai1 in non transfected cells (control), in cultures transfected with scrambled RNA (scramble); siRNA for: STIM1; STIM2; Orai1; Orai2; and for Orai3. Analysis was based on the ΔΔCt method and corrected on β-actin expression (n=4-5).

* b) Western blot showing significant downregulation of STIM1 (left, 49.8 ± 4.5%; n = 3. °p<0.05); and Orai1 proteins (right, 59.6 ± 7.1%; n = 4. °°p<0.01) in primary culture of coronary SMC, 72 hours after siRNA transfection.

* c) Left panel shows representative traces illustrating quantitative changes (F₃₄₀/F₃₈₀) in intracellular Ca²⁺ concentration in individual SMC. Cells were treated with thapsigargin (TG, 2 µM) for 3-4 minutes in the absence of extracellular Ca²⁺, and then 2 mM Ca²⁺ was added as indicated. Traces are for SMC transfected 72 hours prior to the experiments with: scramble siRNA (scramble); STIM1 siRNA (siSTIM1); or Orai1 siRNA (siOrai1). Right panel, illustrates the average changes in fura-2 ratio in individual SMC following the addition of extracellular Ca²⁺ in experiments illustrated in left. “basal” is for Ca²⁺ entry in untreated cells, and “control” is for non transfected cells treated with thapsigargin. The summary data are from 4-6 different transfections. (*) denotes significance vs. control and scramble at p<0.01.
Online figure II.

a) Western blots and densitometry showing significant downregulation of STIM1 (49.9 ± 4.4%, n = 3) and Orai1 (55.4 ± 9.4%, n = 3) proteins, 48 hours after siRNA transfection of coronary artery. *p<0.05.

b) Representative traces of vascular tone from three independent rings transfected with scramble siRNA (scramble, black trace), with siRNA for Orai1 (siOrai1, blue trace), and with siRNA for STIM1 (red trace). Phenylephrine (PE, 0.1 mM) was applied as indicated, then washed out for 30 min. High-KCl (60 mM) was added as a control.

c) Shows the summary data of similar experiments as b. Values are normalized to high-KCL responses and are means ± s.e.m. from 6-10 different experiments.

Online figure III.

Immunofluorescence staining shows localization of iPLA$_2$ (green) and STIM1 (iPLA$_2$) in SMC stained with anti-rabbit iPLA$_2$, and anti-mouse STIM1 antibodies. Images are for untreated SMC (control) and for SMC treated with thapsigargin (TG, 2 μM). Thapsigargin addition induces STIM1 redistribution in small punctae which show high co-localization with iPLA$_2$ as indicated by yellow color in merged images.

Online figure IV.

a) Immunofluorescence staining with specific antibodies shows localization of iPLA$_2$ (green) and STIM1 (red) in SMC. Merged images are for untreated SMC (control) and for cells treated 3-4 minutes with Urotensin-II (UII, 50 nM). Co-localization occurred only after Urotensin-II addition as indicated by yellow color.

b) Graphs show the fluorescence intensity indicating co-localization of STIM1 (red) and iPLA$_2$ (green) signals in different cross-section of the cell (see white line in merged images).

Online figure V.

a) Representative traces and summary data illustrating Ca$^{2+}$ entry in SMC treated with lysophosphatidylcholine (LPC, 300 nM) for 3-4 minutes in the absence of extracellular Ca$^{2+}$, and then 2 mM Ca$^{2+}$ was added as indicated. Traces are for SMC treated with LPC in control cells (control), in cells treated with gadolinium (+Gd$^{3+}$, 5 εM), or with 2APB (+2APB, 50 εM) 1-2 minutes
before Ca\(^{2+}\) addition as indicated by *. "basal" is for Ca\(^{2+}\) influx in untreated SMC. (n = large number of cells from 3 primary cultures). \(\#p<0.01\) for treatments vs. control.

b) Example of changes in isometric tension induced by LPC (300 nM) in coronary artery. LPC-induced contractions were antagonized respectively by 50 \(\mu M\) 2APB (n = 7) and by 5 \(\mu M\) gadolinium (Gd\(^{3+}\), n = 5).

c) Bar graph shows summary data of similar experiments as in "b." Values are means ± s.e.m. from 5-7 different experiments. \(\#p<0.01\) for treatments vs. control

Online figure VI.

a) Representative traces and summary data illustrating Ca\(^{2+}\) entry in SMC treated with lysophosphatidylcholine (LPC, 300 nM) for 3-4 minutes in the absence of extracellular Ca\(^{2+}\), and then 2 mM Ca\(^{2+}\) was added as indicated. Traces are for SMC treated with LPC in control cells (control), in cells preincubated 10 minutes with U73122 (20 \(\mu M\)) to inhibit PLC (U73122 + LPC). "basal" is for Ca\(^{2+}\) influx in untreated SMC. (n = 40-50 cells from 4 primary cultures).
TG

Ca^{2+} influx (D ratio)

0 min
1 min

**P = 0.003**

Figure I
Figure II

a) Western blot analysis showing the expression levels of STIM1, Orai1, and actin/tubulin in control, scramble, and siSTIM1/siOrai1 groups. The density values are represented as bars with error bars. The density of STIM1/actin is shown with a *p<0.01 significance level.

b) Graph showing the PE-induced contraction in response to KCL treatment with or without siOrai1 and siSTIM1. The contraction is measured in mN with a time scale of 10 min.

c) Bar chart illustrating the PE-induced contraction (% of KCL response) in scramble, siSTIM1, and siOrai1 groups. The p-value for siOrai1 compared to scramble is *p<0.01.
Figure III
Figure IV

(a) Control vs. + Urotensin-II

(b) Fluorescence intensity profiles for Stim1 and iPLA2 under control and + Urotensin-II conditions.
**Coronary SMC**

![Diagram of Coronary SMC with LPC and calcium flux](image)

**Coronary vessel**

![Diagram of Coronary vessel with LPC and calcium flux](image)

**LPC**

![Graph showing vasoconstriction](image)

*Figure V*
Figure VI

- Basal 
- U73122 
- Ca\(^{2+}\) influx (Δratio)

**Graph:**
- Time, min
- **0Ca\(^{2+}\) EGTA**
- **Ca\(^{2+}\)**
- **LPC**
- **U73122 + LPC**

**Bar Graph:**
- LPC
- Ca\(^{2+}\) influx (Δratio)
- Basal
- +U73122