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Abstract—Some small molecular weight peptides possess potent vasoactive properties. Herein we have identified the laminin nonapeptide (LNP) CDPGYIGSR as a novel vasoconstrictive agent. Isometric force measurements revealed that LNP induced vasoconstriction in small and large murine arteries in a dose-dependent fashion; LNP also increased vascular tone in human mammary arteries. The vasoactive response was specific for the nonapeptide, because neither scrambled nor very similar peptide sequences modulated vascular tone. As an underlying mechanism we found in $[Ca^{2+}]_i$ imaging experiments that the nonapeptide induced transmembrane $[Ca^{2+}]_i$ influx in vascular smooth muscle cells. Patch clamp experiments showed that LNP activated nonselective cation channels, causing depolarization of the membrane potential and opening of L-type Ca^{2+} channels. The functional effect of LNP was also assessed with catheter measurements in mice in vivo and confirmed vasoconstriction. This effect was restricted to the systemic circulation, because measurements with the perfused lung system demonstrated that LNP did not alter vascular tone in pulmonary arteries. Thus, LNP is a vasoconstrictor in mouse and human arteries, and its vasoactivity is restricted to the systemic vasculature. (***Hypertension*****. 2012;59:1256-1262.)** ● **Online Data Supplement**

Key Words: cell signaling ■ ion channels ■ peptides ■ smooth muscle ■ vasoconstriction

The identification of vasoactive substances is of high interest for a better molecular understanding of the regulation of vascular tone and for the design of novel therapeutic agents. Different small peptides (eg, endothelin, angiotensin II, and bradykinin) have been found to exert potent vasomodulatory functions.[1,2] Most of these peptides are derived from larger molecules that have no effect on vascular tone and are processed to vasoactive molecules via proteolytic cleavage. These can either have vasoconstrictive (endothelin or vasopressin) or vasorelaxant (bradykinin) properties and also exert other cell biological functions; for example, endothelin and angiotensin II stimulate proliferation of smooth muscle cells.[3]

The 9 amino acid peptide CDPGYIGSR, which is part of the LEb domain in the β1 chain of laminin, has been shown to affect vascular growth.[4] In addition, the peptide was also suggested to bind to the 67-kDa laminin receptor,[5] resulting in the inhibition of metastases formation.[6] The minimum sequence necessary for these effects was identified as the laminin pentapeptide YIGSR that also promoted vascular tube formation[5,7] and could inhibit shear stress-induced increase in endothelial NO expression.[8] In addition, laminin was also reported to modulate L-type Ca^{2+} channels.[9] Because laminin and its β1 chain were shown to be in close contact with the endothelium and the smooth muscle layer of vessels[10] and a laminin fragment containing laminin nonapeptide (LNP) was found to be increased in blood of patients experiencing diabetes mellitus,[11] we explored whether LNP has direct impact on vascular tone. Indeed, other extracellular matrix-derived proteins, such as endostatin and elastin peptides, have been shown to be potent vasoactive compounds that not only affect vascularization but also vascular tone regulation.[12,13]

In the present study we have, therefore, investigated the impact of the LNP CDPGYIGSR on vascular tone and found that it induced vasoconstriction in murine and human systemic but not pulmonary arteries. This effect was mediated by depolarization of vascular smooth muscle cells via nonselective cation channels (NSCCs) and transmembrane Ca^{2+} influx through L-type Ca^{2+} channels and could be also found in vivo.

Methods

Please see the online-only Data Supplement.

Results

The laminin nonapeptide CDPGYIGSR (LNP) induces vasoconstriction in resistance arteries and the aorta in a dose-dependent manner. First, we assessed the effect of LNP on vascular tone in isometric force measurements. These exper-

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From the Institute of Physiology I (D.W., M.M., J.C.H., B.K.F.), University of Bonn, Bonn, Germany; Institute for Oral and Musculo-skeletal Biology (M.K.), Center for Biochemistry, Medical Faculty, University of Cologne, Cologne, Germany.

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Correspondence to Bernd K. Fleischmann, Institute of Physiology I, University of Bonn, Sigmund-Freud-Str 25, 53127 Bonn, Germany. E-mail bernd.fleischmann@uni-bonn.de

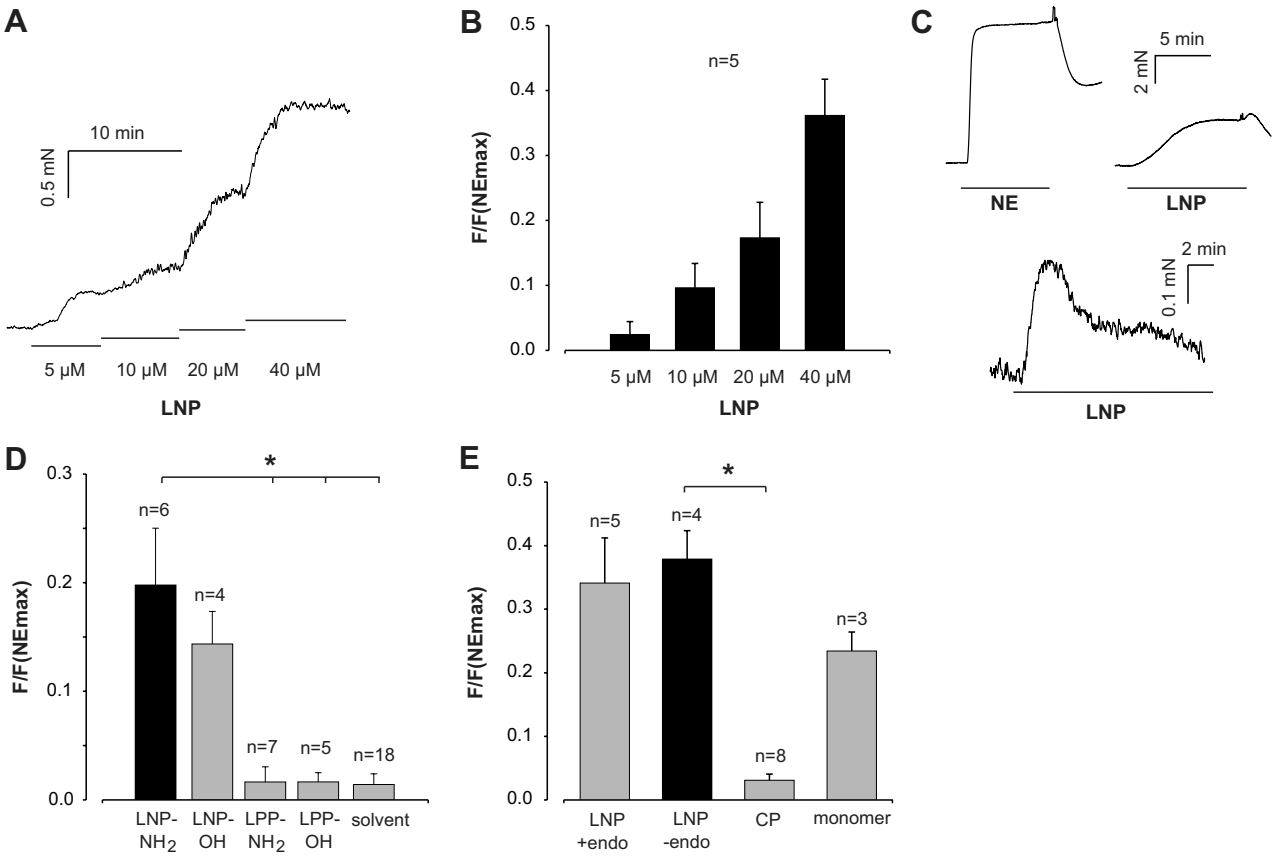

Figure 1. CDPGYIGSR (laminin nonapeptide [LNP]) induces vasoconstriction via the smooth muscle layer. **A** and **B**, LNP evoked a dose-dependent increase of vascular tone in isometric force measurements of mouse-tail artery; the LNP response was normalized to the maximal contraction obtained with norepinephrine (NE). **C**, Original traces of isometric force measurements showing NE (10 μmol/L)- and LNP (40 μmol/L)-induced vasoconstriction in mouse-tail arteries (**top**) and also in the human *Arteria mammaria* (**bottom**). **D**, The amide form of the LNP (LNP-NH_2) proved slightly more active than LNP-OH, whereas the laminin pentapeptide YIGSR (LPP) had no effect. **E**, Removal of the endothelium (-endo) did not affect LNP-induced vasoconstriction. The scrambled control peptide YGRDSGPIS (CP) had no effect, and monomeric LNP evoked a vasoconstrictive response of similar magnitude compared with LNP-endo. *$P<0.05$.

iments revealed that LNP induced a dose-dependent vasoconstriction in the tail artery of mouse (Figure 1A and 1B). The LNP-induced vasoconstriction was found to be sustained for minutes and highly reproducible; Other than tail arteries, we also tested LNP in large arteries, such as the aorta, and found a similar response (data not shown), proving the general vasoactivity of the molecule. In all of the following experiments, we used a high peptide concentration of 40 μmol/L to obtain a strong vasoconstrictive response.

We also explored the vasoactive potential of LNP on human mammary arteries. Similar as in mouse arteries (Figure 1C top), LNP enhanced vascular tone in human vessels (n=3), but the vasoconstrictive response was smaller than in mouse (Figure 1C, bottom). When different forms of LNP were applied in isometric force experiments, we found, similar to an earlier report,[6] that the amide (LNP-NH_2) was slightly more active than the carboxylic acid form (LNP-OH), although the difference did not reach significance (Figure 1D); this could be related to the neutralization of the negative charge on the arginine.[5] Importantly, in contrast to results obtained from cell biological assays, we could not find any vasoactivity of the laminin pentapeptide YIGSR even at high concentrations (160 μmol/L). This was true for both the amide (YIGSR-NH_2) and the carboxylic acid forms (YIGSR-OH; Figure 1D). Because of its more prominent vasoactive effect, we used for all further experiments the amide form of LNP.

We next sought to identify the cell type responsible for the LNP-induced vasoconstriction and removed the endothelium of mouse tail arteries. We found that the vasoconstriction by LNP was very similar in intact and denuded arteries, clearly indicating that it is mediated via a direct effect onto the smooth muscle layer (Figure 1E).

We also assessed the specificity of the response to LNP and applied the scrambled control peptide YGRDSGPIS (40 μmol/L). Other than the altered sequence of amino acids, this peptide differs only by a single substitution of a cysteine residue with a serine that should prevent formation of disulfide bonds. This peptide displayed a complete loss of the biological effect in endothelium-denuded tail arteries, clearly proving that the physiological response is highly specific for the amino acid sequence of LNP. We also examined whether dimer formation of the molecule is of relevance and, therefore, reduced disulfide bonds. The resulting monomers elicited a vasoconstriction that was similar to the nonreduced peptide (Figure 1E), proving that the biological activity of the molecule is independent from its secondary structure.

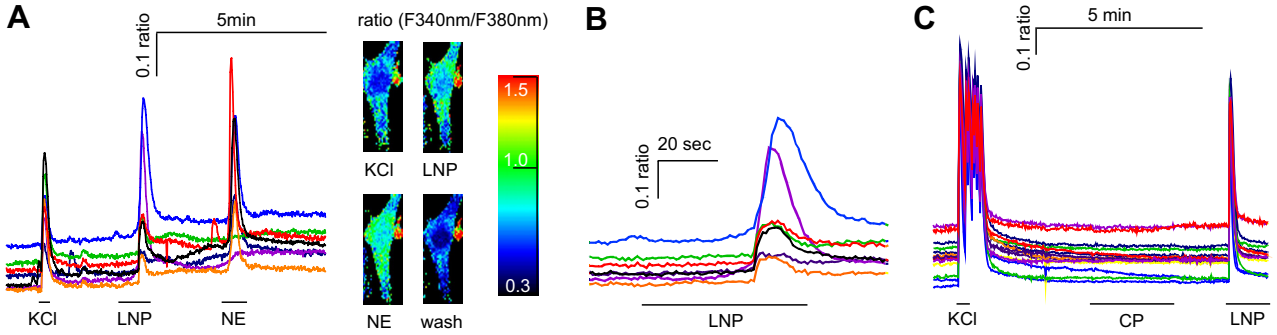

Figure 2. Laminin nonapeptide (LNP) induces $[Ca^{2+}]_i$ increase in vascular smooth muscle cells (VSMCs). **A**, VSMCs were loaded with the Ca^{2+}-dye fura-2 AM for $[Ca^{2+}]_i$ imaging experiments. **Left**, Original traces of a representative experiment of VSMCs from tail arteries. LNP evoked an increase of $[Ca^{2+}]_i$ similar to KCl and norepinephrine (NE). A high ratio (F340 nm/F380 nm) indicated increased $[Ca^{2+}]_i$ concentration, and each trace represents a single cell. **Right**, Pseudocolor picture depicting the F340 nm/F380 nm ratio of the cell represented by the blue line. **B**, Magnification of the response to LNP in **A**. $[Ca^{2+}]_i$ elevation was sustained after application of LNP. **C**, The control peptide (CP) elicited no change of $[Ca^{2+}]_i$.

LNP Binds to the Cell Membrane and Vasoconstriction by LNP Is Specific for the Respective Amino Acid Sequence

Our data reveal that LNP induced vasoconstriction via the vascular smooth muscle layer. To test whether LNP acts either via a membrane receptor-dependent or an intracellular mechanism after uptake of the peptide into vascular smooth muscle cells (VSMCs), we labeled the cell membranes of A7r5 cells, a well-characterized embryonic VSMC line, with a rhodamine-bound lectin that binds to the carbohydrate groups on cell membranes. Thereafter, the cells were incubated with fluorescein isothiocyanate–coupled LNP (40 μmol/L) for $\leq$40 minutes. Fluorescence images showed colocalization of lectin and LNP, indicating that LNP is not taken up by the cells and that the biological response is mediated via a cell surface receptor (Figure S1A and S1B, available in the online-only Data Supplement).

Next, we wanted to identify the amino acid sequence within LNP, which is responsible for vasoconstriction. CDPGYIGSR is specific for the murine and human β1 chains of the laminin molecule. Because very similar amino acid sequences can also be found in other proteins, we experimentally determined the minimal sequence required to maintain the vasoconstrictive response. This approach was taken to rule out that similar peptide sequences contained in other proteins but laminin could reproduce the biological effect. For this purpose, a blast analysis of the peptide sequence was performed yielding 4 similar peptides that are conserved in the mouse and rat. These were tested in isometric force measurements of tail artery, but none evoked a similar biological response as LNP (CDPGYIG [laminin β1 conserved in rat] 6.3±2.9%, n=10; CAPGYIG [MEGF12] −1.5±1.0%, n=4; CDPGYRGK [scavenger protein] 8.1±3.3%, n=4; CDPGY [conserved in several proteins] −4.0±7.4%, n=4; all 40 μmol/L). Thus, the vasoconstriction by LNP is specific for its amino acid sequence.

LNP Evokes an Increase of $[Ca^{2+}]_i$ in VSMCs

To further investigate the signaling mechanism underlying the LNP-induced vasoconstriction, $[Ca^{2+}]_i$ imaging experiments were performed. We used A7r5 cells and freshly isolated VSMCs from murine tail arteries. Positive immunostainings against vimentin, α-smooth muscle actin, and desmin corroborated the successful isolation of VSMCs (Figure S2).

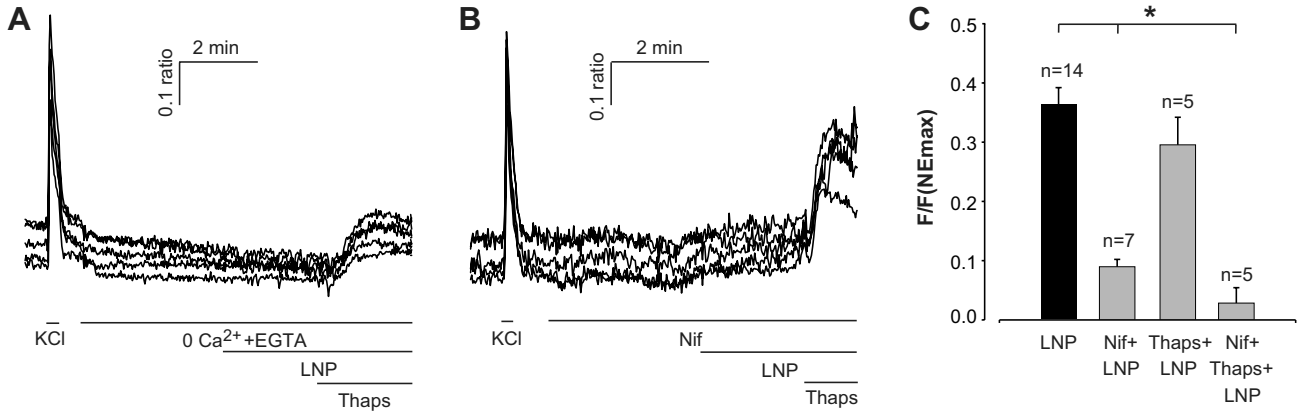

Figure 3. Laminin nonapeptide (LNP) induces transmembrane Ca^{2+} influx and vasoconstriction via L-type Ca^{2+} channels. **A**, Removal of extracellular Ca^{2+} (0 mmol/L Ca^{2+}+2 mmol/L EGTA) prevented elevation of $[Ca^{2+}]_i$ by LNP, whereas the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) blocker thapsigargin (Thaps) could still increase $[Ca^{2+}]_i$ via release from intracellular stores. **B**, The L-type Ca^{2+} channel inhibitor nifedipine (Nif) also blocked LNP-induced $[Ca^{2+}]_i$ increase; thapsigargin again proved that the intracellular Ca^{2+} stores were not depleted. **C**, LNP-induced vasoconstriction was strongly reduced in the presence of nifedipine vs LNP alone. Pretreatment with thapsigargin had no effect on vascular tone. *$P<0.05$.

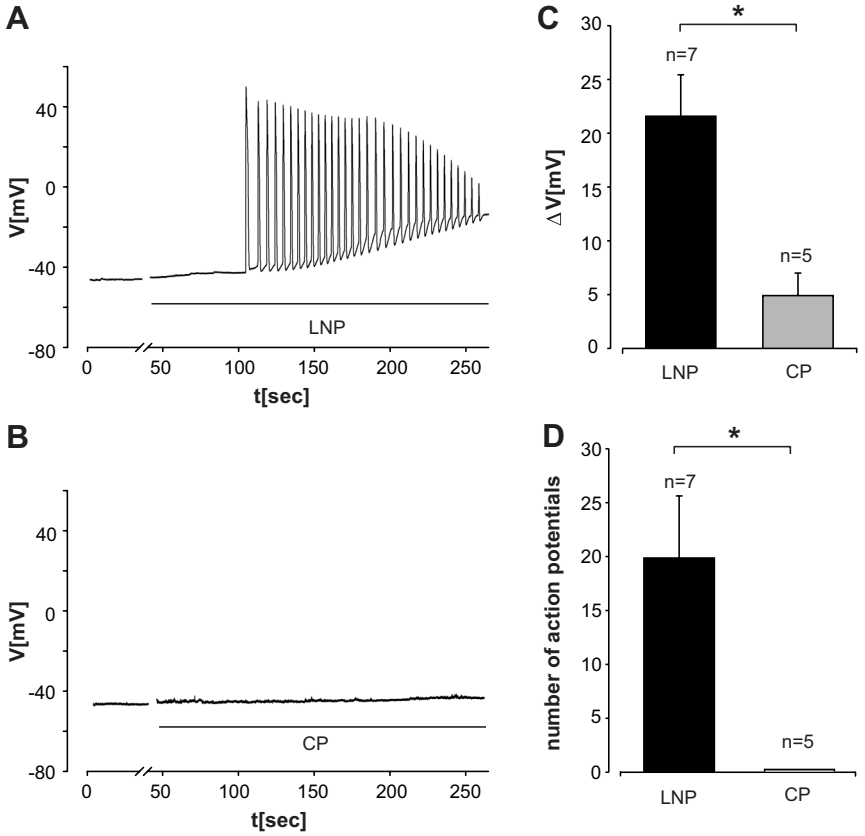

Figure 4. Laminin nonapeptide (LNP) evokes depolarization and action potential generation in vascular smooth muscle cells (VSMCs) in patch clamp experiments. **A**, LNP induced a prominent depolarization and resulting action potential generation in A7r5 cells in the current clamp mode, whereas the control peptide (40 μmol/L; CP; **B**) had no effect. Note break in the traces to omit artifacts during LNP application. **C**, Statistics of effects of LNP and CP on membrane potential in A7r5 cells. **D**, Statistics of effects of LNP and CP on action potential generation. *$P<0.05$.

Only cells responding to exposure of KCl (100 mmol/L) or norepinephrine (10 μmol/L) with an increase of $[Ca^{2+}]_i$ were considered functionally intact and used for the experiments. Application of LNP (40 μmol/L) induced elevations of $[Ca^{2+}]_i$ in the large majority of A7r5 and freshly isolated VSMCs with a response rate of ≈80% (n=13 experiments; Figure 2A). The $[Ca^{2+}]_i$ signal showed a fast rise, and this increase persisted throughout the application of LNP (Figure 2B), similar to the contraction studies. In contrast to LNP, the control peptide (YGRDSGPIS; 40 μmol/L) did not alter $[Ca^{2+}]_i$ (Figure 2C; n=5 experiments). Thus, vasoconstriction by LNP appears to be evoked by a sustained increase of $[Ca^{2+}]_i$ in VSMCs. Next, we examined whether the $[Ca^{2+}]_i$ increase was generated by release from intracellular stores and/or transmembrane Ca^{2+} influx. For this purpose we assessed the effect of LNP in Ca^{2+}-free (0 mmol/L Ca^{2+}, 2 mmol/L EGTA) solution. Under these conditions, LNP did not induce an increase of $[Ca^{2+}]_i$, whereas the sarcoendoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (10 μmol/L) was still able to elevate $[Ca^{2+}]_i$ (Figure 3A; n=4 experiments and 48 cells), proving that the intracellular Ca^{2+} stores were not fully depleted. Furthermore, application of the L-type Ca^{2+} channel inhibitor nifedipine (10 μmol/L) abolished completely the LNP-dependent increase of $[Ca^{2+}]_i$ (n=10 experiments and 64 cells; Figure 3B). These data suggest that the LNP-induced increase of $[Ca^{2+}]_i$ occurs via transmembrane influx of Ca^{2+} through L-type Ca^{2+} channels. The findings were also corroborated in isometric force measurements, where nifedipine strongly reduced vasoconstriction by LNP (Figure 3C). To further confirm the single cell data, entire vessels were preincubated with thapsigargin, which did not affect the LNP-induced vasoconstriction; similarly, the combined application of nifedipine and thapsigargin did not further reduce vasoconstriction when compared with nifedipine alone (Figure 3C). LNP-induced vasoconstriction was also strongly attenuated in response to application of the protein kinase C (PKC) inhibitors Gö6983 (4 μmol/L; Gö6983+LNP, 12.1±4.3%, n=6, versus LNP; $P<0.05$), Gö6976 (4 μmol/L), and bisindolylmaleimide I (4 μmol/L; data not shown). Thus, our experiments demonstrate that the LNP-induced vasoconstriction is mediated via activation of L-type Ca^{2+} channels and transmembrane influx of Ca^{2+} leading to the ensuing increase of $[Ca^{2+}]_i$ and that this process is also modulated by PKC.

LNP Depolarizes the Membrane Potential by Activating NSCCs

The activation of L-type Ca^{2+} channels in VSMCs can either be evoked by direct modulation of Ca^{2+} channels or depolarization of the membrane potential. We examined this aspect in A7r5 cells with help of the patch clamp technique. First, we determined whether LNP modulated L-type Ca^{2+} current directly but could neither observe changes of the peak current amplitude (n=4) nor of the IV relationship (n=2; data not shown). We, therefore, probed the LNP effect using perforated patch in the current clamp mode and found a prominent depolarization of the membrane potential (Figure 4A and 4C) by 21.6±3.1 mV (n=7). The depolarization led to action potential generation in most cells (19.3±3.7, n=7; Figure 4A and 4D). Depolarization and action potential

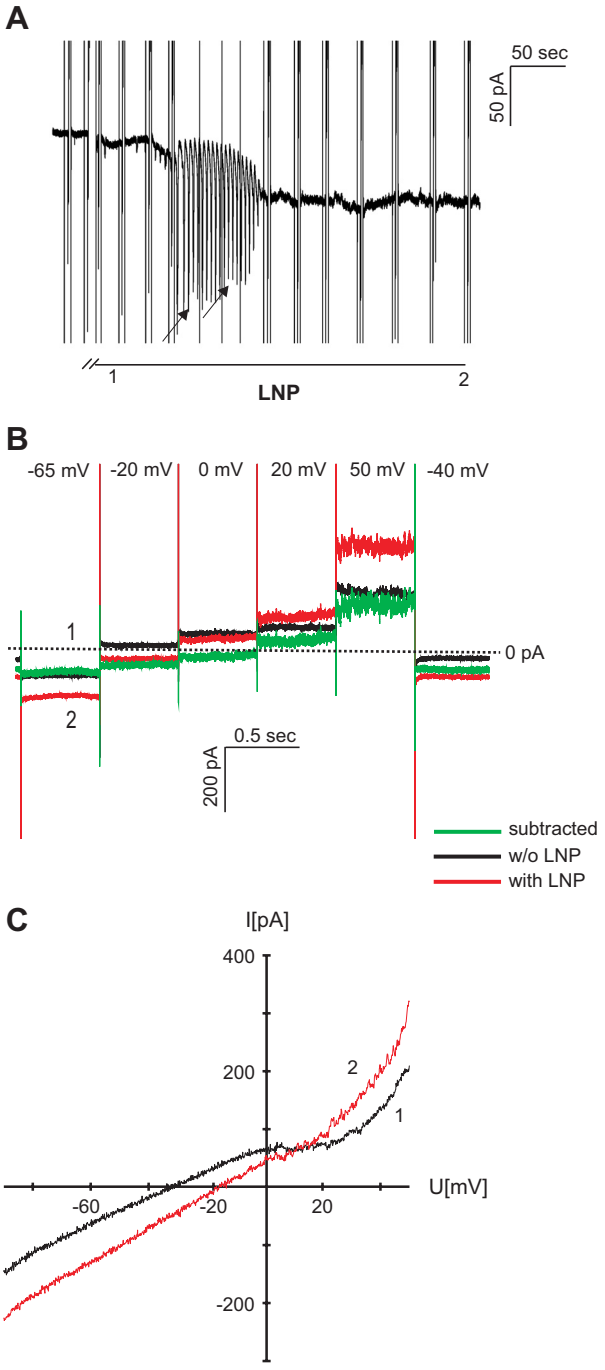

Figure 5. Laminin nonapeptide (LNP) activates nonselective cation channels in vascular smooth muscle cells (VSMCs). **A**, Application of LNP evoked a sustained inward current and intermittent action potentials (**arrows**) in an A7r5 cell held at −40 mV in the voltage clamp mode; intermittent ramp depolarizations (−90 to +80 mV; 150 ms) and voltage steps (500 ms) to the clamp potentials indicated in **B** were applied. The break sign below the current trace indicates that recordings during LNP application are omitted. **B**, Voltage steps obtained before (black; time point 1 in **A**) and after application of LNP (red; time point 2 in **A**); subtraction of the respective current amplitudes (2–1) yielded LNP-induced currents at the different step potentials (green). **C**, Ramp recordings without (w/o) and with LNP, numbers (1,2) indicate time points in the curve at **A** (ramps ranging from −80 to +50 mV are shown).

generation were specific for application of LNP, because the control peptide YGRDSGPIS was without effect on membrane potential (Figure 4B and 4C). In addition, we could exclude a contribution of the Na^+-Ca^{2+} exchanger, because iso-osmolar replacement of extracellular Na^+ by Li^+ did not alter the LNP-evoked depolarization of the membrane potential (data not shown).

To identify the mechanism responsible for the LNP-dependent depolarization of the membrane potential, cells were held in the whole-cell voltage clamp mode close to their physiological membrane potential at −40 mV. Intermittent ramp (−90 to 80 mV; 150 ms) and voltage step depolarizations (−65, −20, 0, 20, and 50 mV; 500 ms) were applied. Application of LNP induced long-lasting inward currents and action potentials (Figure 5A), which became clearly visible as spiking inward currents. The step potentials were measured and a linear regression analysis performed for each cell, yielding an average reversal potential of +1.5±4.2 mV (n=4; Figure 5B), which was close to the calculated equilibrium potential for monovalent cations (≈1.8 mV). Ramp depolarizations applied under control conditions and in the presence of LNP also yielded a reversal potential close to 0 mV (Figure 5C).

Thus, LNP activates NSCCs causing depolarization. We tried to characterize the molecular identity of the LNP-activated NSCCs and, therefore, measured the vasoconstrictive response in transient receptor potential (TRPC) 1,3,6 knockout mice. These mice were chosen because angiotensin II and vasopressin have been reported to activate TRPC 1 and 6 in vascular smooth muscle cells[14,15] and because TRPC 3 activity was found to be increased after TRPC 6 deletion.[16] Isometric force measurements in tail arteries of TRPC 1, 3, and 6 knockout mice demonstrated that the LNP-induced vasoconstrictive effect remained unaltered (Figure S3), indicating that other NSCCs than TRPC 1, 3, and 6 are involved in the LNP response.

The NSCC-dependent depolarization was found to result in L-type Ca^{2+} channel activation, and this is supported by experiments where VSMCs were measured in the current clamp mode and treated with nifedipine. Under these conditions, LNP did not induce action potentials (Figure S4A and S4C), whereas the LNP-dependent depolarization was found to be unaltered in the cells (Figure S4A and S4B). Current clamp experiments with the PKC inhibitor Gö6983 yielded similar effects, because LNP-evoked depolarization remained unaltered, whereas almost no action potentials were observed (LNP+Gö6983: V (before LNP application): −41.4±1.7 mV; ΔV: 14.5±3.7, 0.9±0.6 action potentials; n=8; $P>0.05$ versus LNP).

LNP Specifically Increases Systemic Pressure in Mice In Vivo

Next, we explored whether the intravenous application of LNP also exerted vasoconstrictive action in vivo. For this purpose, left ventricular pressure was monitored via a catheter before and after injection of LNP into the jugular vein of mice. We found that LNP (4 mg/kg in 6 μL of physiological salt solution) evoked an increase of systolic pressure from 82.2±2.8 (pre=LNP) to 91.3±3.7 mm Hg (post-LNP; n=8;

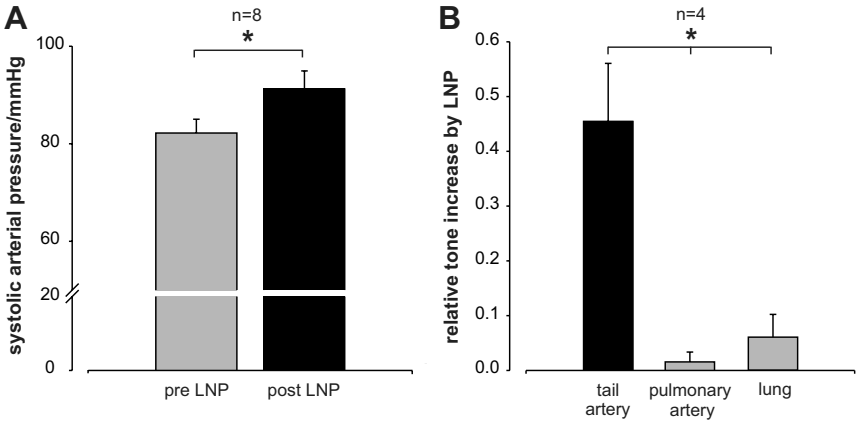

Figure 6. Laminin nonapeptide (LNP) induces vasoconstriction in the systemic circulation in vivo but has no effect on pulmonary arterial tone. **A**, LNP (4 mg/kg) increased systolic arterial pressure on intravenous injection during left ventricular catheter measurements (pre indicates before LNP injection; post, peak systolic values after LNP injection). **B**, LNP increased vascular tone in the tail artery but not in the pulmonary artery and isolated lung; values were normalized to maximal constriction. *$P<0.05$.

$P<0.05$; Figure 6A). The diastolic pressure and the beating frequency of the heart remained unaltered. We also wondered whether LNP modulated vascular tone in the pulmonary circulation and performed isometric force measurements of the large murine pulmonary artery. Our experiments revealed that LNP did not increase vascular tone in large pulmonary arteries (Figures 6B and S5A). We also performed experiments with the isolated perfused lung system to investigate the impact of LNP on the tone of small and very small intrapulmonary vessels. Similar to our findings in large pulmonary vessels, LNP did not evoke a pressure increase in the isolated perfused lung (Figures 6B and S5B). Moreover, $[Ca^{2+}]_i$ imaging in human pulmonary arterial smooth muscle cells confirmed that LNP had no effect on pulmonary smooth muscle cells (only 1 of 157 cells in a total of 5 experiments showed $[Ca^{2+}]_i$ increase; Figure S6). Thus, LNP specifically increases vascular tone in systemic but not in pulmonary arteries.

Discussion

Herein we report that the LNP CDPGYIGSR is a novel vasoconstrictor in murine and human arteries. The vasoconstrictive action of LNP is specific for the systemic circulation, because large and small pulmonary arteries were unresponsive. We found that LNP exerts its biological action on VSMCs and induces an NSCC-dependent depolarization of the membrane potential in these cells. This results in the activation of voltage-dependent L-type Ca^{2+} channels, a sustained increase of cytosolic Ca^{2+}, and vasoconstriction. These findings are in line with earlier reports demonstrating that extracellular matrix peptides are potent regulators of vascular tone.[13,17]

Therefore, and also because LNP has been reported to be cell biologically active in the vascular system,[7] we tested the impact of this molecule on vascular tone. We found that LNP induced a strong vasoconstriction in a sex-independent fashion (males: 29.3±3.4%, n=12, $P>0.05$ versus females) and explored the mechanism underlying this effect. Our studies revealed that LNP does not cross the plasma membrane, suggesting a membrane receptor-dependent pathway. The experimental results imply that it is not the 67-kDa laminin receptor, which provides YIGSR-dependent cell attachment in laminin,[18] because laminin pentapeptide had no effect on vascular tone. It is, therefore, possible that LNP signals via the second known binding site for laminin on the 67-kDa receptor, the so-called G peptide,[19] and/or other unknown laminin binding sites; alternatively, integrins alone or in combination with the 67-kDa receptor could also be involved in LNP signal transduction.

We suspected a critical role for L-type Ca^{2+} channels in the LNP response in VSMCs, because the specific blocker nifedipine prevented the LNP-evoked increase of $[Ca^{2+}]_i$ and the ensuing vasoconstriction. We found that LNP did not directly activate voltage-dependent L-type Ca^{2+} channels but induced depolarization of the membrane potential via activation of NSCCs; these ion channels have been shown to induce depolarization of the membrane potential in VSMCs before.[20] Earlier electrophysiological studies in A7r5 cells or freshly isolated rat aortic smooth muscle cells reported endothelin- or vasopressin-mediated activation of NSCCs, causing depolarization of the membrane potential and Ca^{2+} influx; this was claimed to be attributable to the Ca^{2+} permeability of NSCCs.[21] In the present study we found that the increase of $[Ca^{2+}]_i$ was attributed to depolarization-dependent opening of L-type Ca^{2+} channels, because nifedipine completely blocked the LNP-induced increase of $[Ca^{2+}]_i$, and the measured reversal potential of the LNP-activated NSCCs was close to the calculated reversal potential for monovalent cations, suggesting no or only low permeability to Ca^{2+}.

Because of the relatively slow on-kinetics of the LNP-induced response, we explored the potential involvement of principal intracellular signaling pathways, such as phosphatidylinositol 3-kinase and PKC, which are known to modulate L-type Ca^{2+} channels in vessels.[22] Although the phosphatidylinositol 3-kinase inhibitor LY294002 had no impact on vasoconstriction by LNP (10 μmol/L; n=4; data not shown), PKC inhibition led to a reduction of LNP-induced vasoconstriction and inhibition of action potential generation in VSMCs. These findings are in agreement with earlier reports, showing that inhibition of PKC can prevent activation of L-type Ca^{2+} channels, resulting in a reduction of vascular tone.[23] Beyond the strong vasoconstrictive effect of LNP when exogenously applied, it remains to be seen whether the LNP domain within the native protein can, as suggested by earlier studies, directly interact with VSMCs under physiological or pathophysiological conditions and act endogenously. We have tried to address this experimentally by synthesizing a large 45-kDa fragment of the β1 chain

displaying the correct tertiary structure. In some experiments (n=3), we could observe an increase of $[Ca^{2+}]_i$ in VSMCs on application of this protein (1 μmol/L; data not shown). However, because of its relatively large molecular size, we could not test this protein in other assays, and, therefore, additional studies are needed in the future to provide definitive answers, whether this molecule may also play a role in the pathophysiology of hypertension. Nevertheless, this substance could be potentially useful for therapeutic purposes, because some patients experiencing septic shock or anaphylaxis have been reported to be unresponsive to classic vasoconstrictive agents.[24,25] LNP acts via a different mechanism and specifically elevates systemic pressure in vivo, whereas it is without effect in pulmonary vessels, a pharmacological activity profile reminiscent of vasopressin.[26]

Perspectives

Altogether, we show that the LNP CDPGYIGSR is a novel vasoactive substance in murine and human systemic arteries. It exerts its biological effects via NSCC-dependent depolarization and L-type Ca^{2+} channel activation. This finding is of potential therapeutic importance, because the vasoregulatory effects of LNP are restricted to the systemic circulation.

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Disclosures

None.

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ONLINE SUPPLEMENT

Identification of a novel vasoconstrictor peptide specific for the systemic circulation

Laminin peptide CDPGYIGSR is a new vasoregulator

Daniela Wenzel[1]; Manuel Koch[2], Michaela Matthey[1], Jan C. Heinemann[1], Bernd K. Fleischmann[1]

[1]Institute of Physiology I, University of Bonn, [2]Institute for Oral and Musculo-skeletal Biology, Center for Biochemistry, Medical Faculty, University of Cologne

Corresponding author:
Bernd K. Fleischmann, MD
Institute of Physiology I, University of Bonn
Sigmund-Freud-Str.25
53127 Bonn
Tel-No: xx49/228/6885/200
Fax: xx49/228/6885/201
e-mail: bernd.fleischmann@uni-bonn.de

Materials and Methods

All animal work was performed in 10-12 weeks old female and male CD1 mice and complied with procedures approved by the regional and local Animal Care Committees.

Isometric force measurements

Isometric force measurements involving the use of human arteries were approved by the local Ethics Committee. After informed consent mammary arteries were obtained from 3 male patients undergoing coronary bypass surgery.

Isometric force measurements were performed in a wire myograph as described recently[1,2]. Briefly, mouse tail artery was dissected free of connective tissue and cut into 2-mm-long rings in cold (4 °C) low calcium physiological saline solution (PSS) containing 118 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM NaH_2PO_4, 0.16 mM $CaCl_2$, 10 mM glucose, and 24 mM HEPES, pH 7.4. In some experiments, the endothelium of the vascular rings was removed with a mouse whisker. The vessel was considered as denuded when relaxation upon acetylcholine was below 15% of the submaximal norepinephrine (NE) contraction. Arterial segments were mounted on a small vessel wire myograph (Multi Myograph 610 M, Danish Myo Technology, Aarhus, Denmark). A computer-assisted normalization protocol was performed to pre-stretch vascular rings to $0.9 \times L_{100}$ with L_{100} = diameter mimicking a transmural pressure of 100 mmHg. Rings were equilibrated for 20 min in physiological saline solution composed of 118 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM NaH_2PO_4, 1.6 mM $CaCl_2$, 10 mM glucose, and 24 mM HEPES, pH 7.4. The solution was bubbled with 100% oxygen and heated to 37°C. Before the experiments the tail arteries were maximally contracted with NE (10 μM) and pulmonary arteries with phenylephrine (10 μM). For analysis all forces were normalized to the maximal contraction (e.g. F/F(NEmax)).

Reduction of LNP dimers

To reduce potential disulfide bonds between dimeric LNP the peptide was dissolved in PSS to obtain a concentrated stock solution containing 1 mM β-mercaptoethanol. After 1 hour cysteine residues were prevented from recombining by alkylation with a slight molar excess of iodoacetamide (1.2 mM). Then after the next hour the peptide was diluted to reduce excess reagents and used for isometric force measurements.

Preparation of vascular smooth muscle cells (VSMCs) and cultivation of A7r5

Mouse tail arteries were prepared as described above and cut into small pieces. These were dissociated in collagenase B for 20 min and plated on gelatine-coated coverslips. The cells were cultivated for approximately one week in Dulbecco`s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 20% fetal calf serum (FCS), 1% non-essential amino acid, 0.1% β-mercaptoethanol, 1

% penicillin/streptomycin until they had grown to confluence. A7r5 cells (ECACC) were cultivated in DMEM + 10% FCS and 1% sodium pyruvate and used until passage 10. Then, the cells were subjected to immunostaining, $[Ca^{2+}]_i$ imaging or patch clamp experiments.

Immunohistochemistry

Immunohistochemistry was performed as described before[3,4]. After fixation of the cells with 4% paraformaldehyde immunostaining was performed using primary antibodies against vimentin (1:1000, Chemicon, Temecula, CA, USA), α-smooth muscle actin (1:800, Sigma-Aldrich) and desmin (1:200, Progen Biotechnik, Heidelberg, Germany). Primary antibodies were visualized by secondary antibodies conjugated with Cy3 (1:400, Dianova, Hamburg, Germany) and nuclei were stained with Hoechst3342 (1:1000, Sigma-Aldrich, Taufkirchen, Germany).

Internalization experiments

The membranes of A7r5 cells were labeled with Griffonia simplicifolia lectin conjugated with Cy3 (Vector Laboratories, Burlingame, CA, USA), nuclei were stained with Hoechst. Then the cells were incubated for up to 60 min with the LNP which had been labeled with the FITC fluorochrome (Genscript Corporation, New Jersey, USA). Pictures were taken with an inverted microscope (Axiovert 200, Carl Zeiss Microimaging GmbH, Oberkochen, Germany) equipped with an Apotome.

$[Ca^{2+}]_i$ imaging experiments

VSMCs from tail arteries or A7r5 cells were loaded for 20 min with the cell-permeable, ratiometric Ca^{2+}-dye fura-2 AM. The cells were transferred to the stage of an inverted microscope and perfused with PSS. Excitation light (340 nm, 380 nm) was generated by a computer-controlled monochromator. The fluorescence of the cells was imaged through a 510 nm longpass filter and recorded by a CCD camera. Pictures were acquired at 0.7 Hz and 30 ms exposure time using the TILLVision software (TILL Photonics, Germany). All pharmacological agents were applied through a hand-made pipette connected to a gravity-driven perfusion system. Only cells responding to KCl (100 mM) or NE (10 µM) treatment with $[Ca^{2+}]_i$ elevation were used for the experiments. All experiments were performed at room temperature. For analysis the ratio of emitted light intensities after 340 nm and 380 nm excitation was calculated.

Patch clamp experiments

In the current clamp mode A7r5 cells were measured in perforated patch whole cell recordings. Therefore, pipettes with resistances from 3-5 MΩ were dipped for 10 sec in internal solution containing (in mM): KCl 50, Kgluconat 80, EGTA 10, Hepes 10, MgATP 3, $MgCl_2$ 1, pH 7.35 (KOH) and then backfilled with the same solution containing 200 µg/ml nystatin. The external solution consisted of (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 10, Glucose 10, pH 7.4 (NaOH). No current was injected and only cells with a membrane potential below -35 mV were analyzed. In the voltage clamp mode whole cell configuration was

used and Mg^{2+} was omitted from the solutions to prevent divalent cation block. Cells were held at a potential of -40 mV and alternating ramp protocols (-90 to 80 mV, 150 ms) and steady state I-V relationships (–65 mV, -20 mV, 0 mV, 20 mV and 50 mV, 500 ms) were recorded. Data were sampled at 1 kHz and filtered with 0.5 kHz. The LNP and the control peptide (CP) were added to the bath solution during the measurements. For the determination of the reversal potential of the LNP-induced inward current, we used the voltage step protocol because of better reproducibility than ramp depolarizations probably due to the activation of time- dependent conductances. The reversal potential of 4 cells was extrapolated by fitting regression lines to the individual current/voltage relationships without and with LNP and determining the intersections. Then, the average value was calculated and compared to the calculated equilibrium potential for monovalent cations. The LNP effect was determined at the time point of the strongest depolarization.

Isolated perfused lung experiments

Isolated perused lung experiments were performed as reported previously with the setup of Hugo Sachs Elektronik (March-Hugstetten, Germany)[2]. Briefly, after mice were sacrificed the trachea was cannulated and lungs were ventilated at 80 breath/min. Then mice were exsanguinated and the pulmonary artery and the left ventricle were cannulated to allow perfusion of the pulmonary vasculature. Ventilation was performed at negative pressures (-2 to -10 cmH_2O). For perfusion a roller pump at a flow rate of 1 ml/min was used. LNP was applied in PSS solution (see above) and changes in arterial pressure were monitored by a pressure transducer connected to the cannula in the pulmonary artery. Values were normalized to maximal contraction obtained by 10 μM serotonin (5-HT).

Left ventricular catheter measurements

All animal work was performed in female CD1 mice and complied with procedures approved by the regional and local Animal Care Committees. For in vivo measurements of the systemic blood pressure mice were anesthetized with 1.5 % isoflurane and 0.6 l/min O_2. Then, animals were fixated on a heating plate (37 °C). The skin was cut from mandible to sternum. The right carotid artery and the left jugular vein were dissected free of connective tissue. Two ligations were placed around the proximal and distal ends of the carotid artery. Then the proximal one was pulled tight and stretch was applied on the distal one. A small incision was made near the proximal end and the pressure catheter (1 F, Millar) was inserted into the left ventricle via the carotid artery. This catheter continuously monitored pressure via the Millar Aria 1 system connected to a PowerLab A/D converter (ADInstruments, Spechbach, Germany). LNP (4 mg/kg in 6 μl) was injected into the left jugular vein with a Hamilton syringe connected to a tube and a small needle. For analysis the maximal increase of systolic pressure was used. Only experiments with systolic pressure increase were used for analysis (n=8), experiments with obvious technical problems and/or pressure decease were omitted. Analysis was performed with the blood pressure module of the LabChart 7 pro software (ADInstruments).

Chemicals

All chemicals were purchased from Sigma-Aldrich with the exception of the peptides CDPGYIGSR and YIGSR (Bachem, Bubendorf, Switzerland) and the custom-made peptides that were synthesized by GenScript Corporation (New Jersey, USA).

For the preparation of stock solutions all drugs were dissolved in the solvent according to the recommendations of the manufacturer and stored in aliquots at -20°C. Prior to the experiments, aliquots were further diluted using the respective solutions, the final DMSO concentration was always less than 0.1%.

Statistical analysis

Data are indicated as mean ± standard error of the mean (SEM). Statistical differences were determined using ANOVA and Bonferroni`s, Dunnet`s test for multiple comparisons or student`s t-test. $P < 0.05$ was considered significant.

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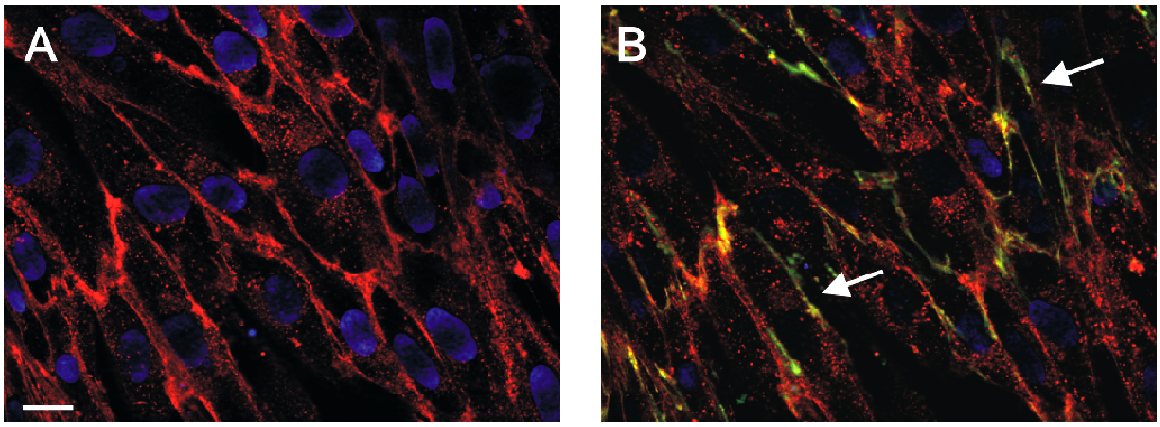

Figure S1: LNP binds to the plasma membrane of vascular smooth muscle cells (VSMCs). (A) Lectin staining (red) of A7r5 cells labels the plasma membrane of the cells, nuclei were stained with Hoechst dye (blue). (B) 20 min after application of the FITC-labeled LNP (green) the peptide co-localized exclusively with the plasma membrane (yellow), no intracellular uptake occurred, bar=20 μm.

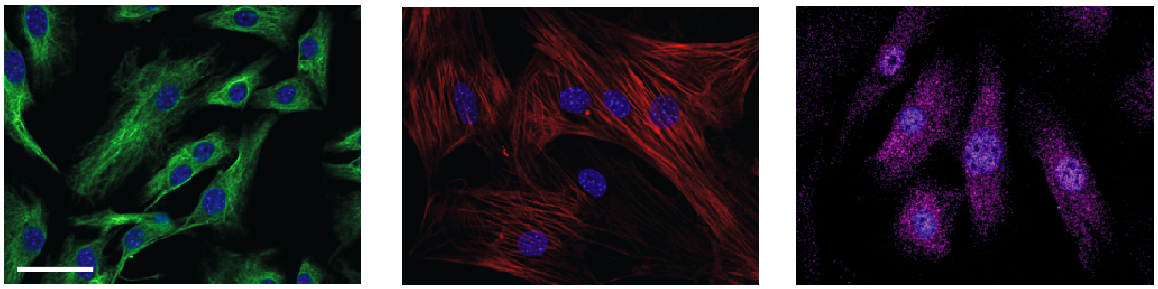

Figure S2: Immunostainings show surface marker expression of tail artery-derived VSMCs. Stainings demonstrated the expression of vimentin (green), α smooth muscle actin (red) and desmin (purple) in VSMCs that were obtained from mouse tail arteries, bar=50 μm.

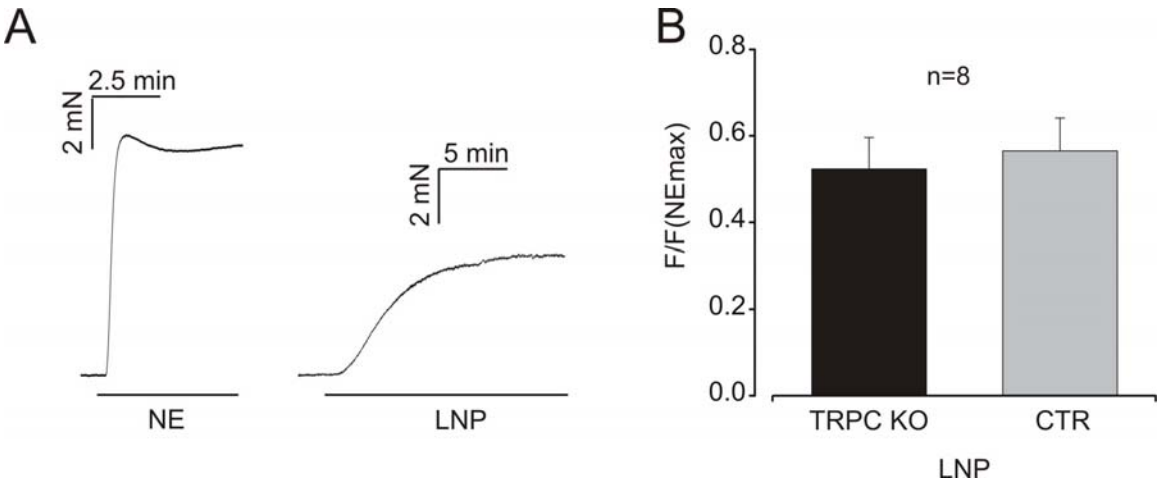

Figure S3: TRPC 1,3,6 are not involved in LNP-induced vasoconstriction. (A) Original traces of isometric force measurements showing NE (10 μM)- and LNP (40 μM)-evoked vasoconstriction in mouse tail arteries of TRPC 1,3,6 KO mice. (B) Statistics of LNP effects in TRPC 1,3,6 KO (TRPC KO) mice compared to control mice (CTR).

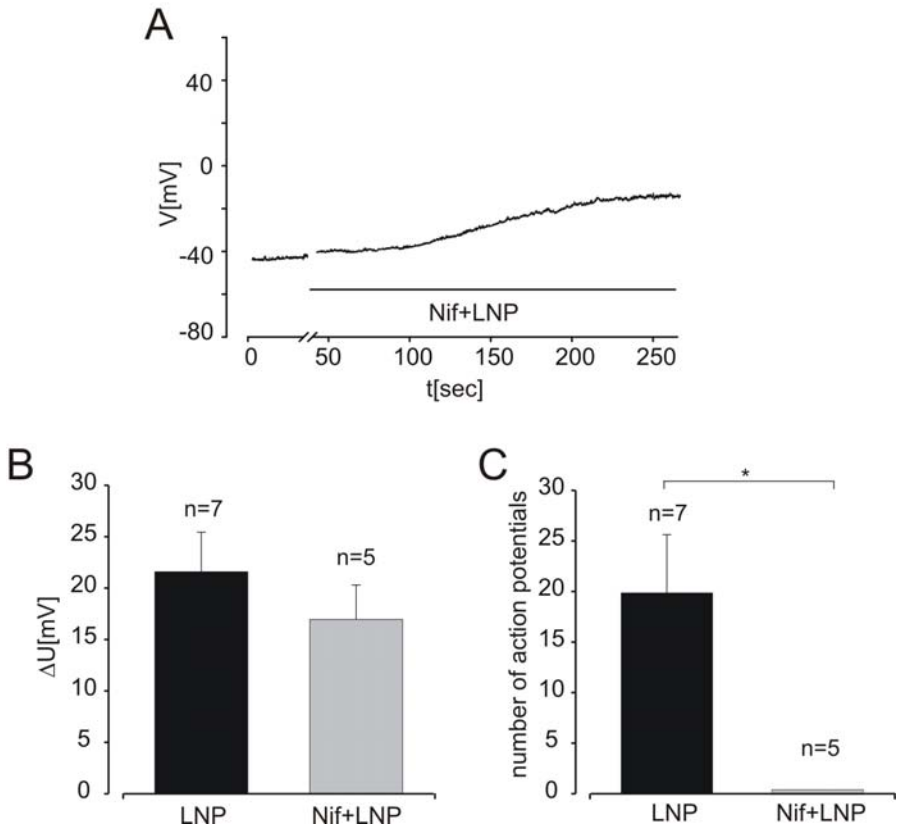

Figure S4: Action potential generation by LNP is mediated via L-type Ca^{2+} channel activation in VSMCs. (A) The L-type Ca^{2+} channel blocker nifedipine (Nif) blocked action potential generation but not depolarization in A7r5 cells, note break in the trace to omit artefacts during LNP application. (B) Statistics of effects of LNP and nifedipine + LNP on membrane potential in A7r5 cells. (C) Statistics of effects of LNP and nifedipine + LNP on action potential generation in A7r5 cells; LNP experiments are the same as shown in Figure 4, *p<0.05.

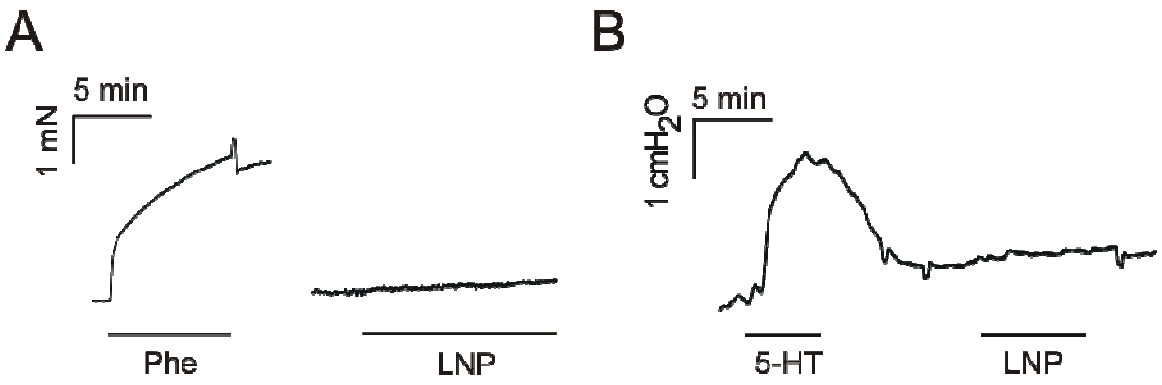

Figure S5: LNP does not affect pulmonary arterial tone. (A) LNP did not elevate the tone of a large pulmonary artery in isometric force measurements. (B) LNP had no effect on pulmonary arterial pressure in the isolated perfused lung model

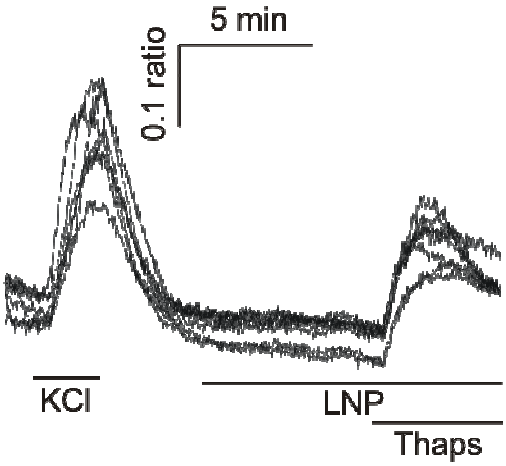

Figure S6: LNP has no effect on $[Ca^{2+}]_i$ in human pulmonary arterial smooth muscle cells (hPASMCs). LNP did not increase $[Ca^{2+}]_i$, whereas the SERCA blocker thapsigargin (Thaps) could still augment $[Ca^{2+}]_i$ via release from intracellular stores.