Activation of neutral sphingomyelinase is involved in acute hypoxic pulmonary vasoconstriction

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Aims The mechanisms involved in hypoxic pulmonary vasoconstriction (HPV) are not yet fully defined. The aim of the study was to determine the role of protein kinase C \(\zeta\) (PKC\(\zeta\)) and neutral sphingomyelinase (nSMase) in HPV.

Methods and results Ceramide content was measured by immunocytochemistry and voltage-gated potassium channel (KV) currents were recorded by the patch clamp technique in isolated rat pulmonary artery smooth muscle cells (PASMC). Contractile responses were analysed in rat pulmonary arteries mounted in a wire myograph. Pulmonary pressure was recorded in anesthetized open-chest rats. Protein and mRNA expression were measured by western blot and RT–PCR, respectively. We found that hypoxia increased ceramide content in PASMC which was abrogated by inhibition of nSMase, but not acid sphingomyelinase (aSMase). The hypoxia-induced vasoconstrictor response in isolated pulmonary arteries and the inhibition of KV currents were strongly reduced by inhibition of PKC\(\zeta\) or nSMase but not aSMase. The nSMase inhibitor GW4869 prevented HPV in vivo. The vasoconstrictor response to hypoxia was mimicked by exogenous addition of bacterial Smase and ceramide. nSMase2 mRNA expression was \(\sim 10\)-fold higher in pulmonary compared with mesenteric arteries. In mesenteric arteries, hypoxia failed to increase ceramide but exogenous SMase induced a contractile response.

Conclusion nSMase-derived ceramide production and the activation of PKC\(\zeta\) are early and necessary events in the signalling cascade of acute HPV.

KEYWORDS
Hypoxic pulmonary vasoconstriction; Neutral sphingomyelinase; Protein kinase C \(\zeta\); Pulmonary arteries

1. Introduction
Hypoxic pulmonary vasoconstriction (HPV) is an adaptive physiological mechanism that optimizes blood oxygen saturation by increasing pulmonary vascular resistance in poorly aerated lung regions, thereby diverting pulmonary blood flow to the better ventilated ones.\textsuperscript{1–7} HPV reflects an intrinsic property of the small pulmonary artery (PA) smooth muscle cells (PASMC) in response to alveolar hypoxia. Generalized alveolar hypoxia associated with altitude, atelectasis, chronic obstructive pulmonary disease, or sleep apnea induces HPV which, if sustained, may lead to pulmonary hypertension and right ventricular failure. On the contrary, failure of HPV, as occurs in adult respiratory distress syndrome, pneumonia, sepsis, or liver cirrhosis is often a critical determinant of ventilation–perfusion mismatch and, hence, hypoxaemia.\textsuperscript{8}

Despite intensive research, the molecular basis of HPV remains one of the most enduring mysteries of cell physiology and several, sometimes contradictory, hypotheses have emerged to explain it.\textsuperscript{1–7,9–12} Nevertheless, there is consensus about the involvement of a putative redox-based \(O_2\) sensor, i.e. mitochondrial electron transport chain\textsuperscript{2,4,5}.

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and/or the membrane NADPH oxidase,\(^5,7\) regulating the activity of effector proteins and there is a large body of evidence indicating a role of the reactive oxygen species (ROS) as signalling intermediates. Voltage-gated \(K^+\) (\(K_V\)) channels are known effector proteins which are inhibited by hypoxia, leading to cell membrane depolarization and opening of voltage-dependent L-type \(Ca^{2+}\) channels.\(^1,7,9\) Additional effectors include twin pore domain \(K^+\) channels whose inhibition also depolarizes the membrane, store-operated \(Ca^{2+}\) channels (SOCs) leading to increased capacitative \(Ca^{2+}\) entry and Rho kinase which is involved in \(Ca^{2+}\)-sensitization.\(^10,12\)

We have reported that protein kinase \(C, \zeta\) (PK\(C_\zeta\)) is involved in the \(K_V\) channel inhibition and the pulmonary vasconstriction induced by the thromboxane \(A_2\) mimetic U46619.\(^13\) The role of this kinase, as well as its adaptor protein p62, was recently confirmed using PK\(C_\zeta\) \(^{-/-}\) and p62 \(^{-/-}\) mice.\(^14\) PK\(C_\zeta\) is directly activated by ceramide,\(^15\) a sphingolipid-derived second messenger molecule. Ceramide is synthesized from membrane sphingomyelin by sphingomyelin phosphodiesterases (SMPD; SMPD1, SMPD2, SMPD3, and SMPD4), also known as acid or neutral sphingomyelinases (aSMase, nSMase1, nSMase2, and nSMase3, respectively) which are activated by multiple membrane receptors and non-receptor stimuli.\(^16\) SMases-derived ceramide production is an attractive candidate mechanism in HPV because aSMase and nSMase are activated by ROS\(^16\) and SMase and ceramide have been shown to inhibit \(K_V\) channels.\(^17,18\)

Herein, we show for the first time that the activation of nSMase and PK\(C_\zeta\) are necessary events in the signalling of HPV.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by our institutional review board. A detailed description of the experimental methods is available in previous studies.\(^13,14,19,20\) Third generation PA (250–450 \(\mu\)m) and mesenteric arteries of similar diameter were isolated from male Wistar rats and smooth muscle cells were enzymatically isolated.

2.1 Solutions and hypoxia

Hepes-buffered solution contained (in mmol/L): NaCl 130, KCl 5, MgCl\(_2\) 1.2, CaCl\(_2\) 1.5, glucose 10, HEPEs 10, pH 7.3, and Krebs solution (in mmol/L): NaCl 118, KCl 4.75, NaHCO\(_3\) 25, MgSO\(_4\) 1.2, CaCl\(_2\) 2.0, KH\(_2\)PO\(_4\) 1.2, and glucose 11 bubbled with 21% \(O_2, 5%CO_2\). For the \textit{in vitro} experiments, hypoxia was induced by bubbling the Hepes solution with \(100\%\) \(N_2\) and the Krebs solution with \(95\%N_2-5\% CO_2\) to achieve an oxygen concentration of \(3-4\%\) (\(24\) \(\pm 1\) Torr) in the chamber. For the \textit{in vivo} experiments, hypoxia was induced by switching the inspiratory input from room air (\(-21\% O_2, normoxia\)) to a 10\% \(O_2-90\% N_2\) gas.

2.2 Ceramic content

Freshly isolated cells adhered to gelatine-coated coverslips were perfused with Hepes solution for 15 min with or without inhibitors and subsequently with hypoxic solution during 0, 1, 3, or 5 min and rapidly fixed with 4% paraformaldehyde. Cells were stained using an anti-ceramide antibody (15B4) and then with donkey anti-rabbit FITC conjugated antibodies. Immunofluorescence was quantified using ImageJ (ver. 1.32j), NIH.

2.3 Contractile responses

PA rings were mounted in a wire myograph in Krebs solution and stretched to give an equivalent transmural pressure of 30 mmHg.\(^21\) Each vessel was exposed to three hypoxic challenges of 10 min duration each, leaving a 45 min incubation period in normoxia between hypoxic challenges. The third hypoxic response was examined after 45 min incubation with vehicle (control) or different inhibitors and the contractile responses were expressed as a percentage of the second exposure to hypoxia.

2.4 Pulmonary arterial pressure

Pressure was recorded with a pressure transducer in anesthetized (100 mg kg\(^{-1}\) ketamine plus 5 mg kg\(^{-1}\) diazepam) open chest rats via a catheter advanced through the right ventricle and placed into the main PA.

2.5 Electrophysiological studies

Membrane currents were recorded using the whole-cell configuration of the patch clamp technique. Cells were superfused with an external \(Ca^{2+}\)-free Hepes solution and a \(Ca^{2+}\)-free pipette (internal) solution containing (mmol/L): KCl 110, MgCl\(_2\) 1.2, Na\(_2\)ATP 5, HEPEs 10, EGTA 10, pH adjusted to 7.3 with KOH. Currents were evoked following the application of 200 ms depolarizing pulses from \(-60\) mV to test potentials from \(-60\) mV to \(+60\) mV in \(10\) mV increments.

2.6 Protein expression

Protein expression was quantified by western blotting using anti-Kv1.5 (Alomone, Israel), anti-PKC\(\zeta\) (Santa Cruz Biotechnology), anti-\(\alpha\)-actin (Sigma) antibodies, or an affinity purified rabbit anti-nSMase2 antibody (prepared by Genscript). The synthetic oligopeptide used for immunization was CRRRHPDEAFDHEVS (identical to the 335–348 amino acids of rat nSMase2 plus an N-terminal cystein and similar to that used by Tani and Hannun\(^22)\).

2.7 Real-time RT–PCR

Total RNA was isolated and purified from arterial homogenates using RNeasy Mini kit (Qiagen). Real-time PCR was performed using a Taqman system (Roche Diagnostics, Mannheim, Germany) in the Unidad de Genomica (Universidad Complutense de Madrid). Specific primers were designed for rat aSMase (right 5'- TTTCGGGAGCTCTG AGA-3' and left 5'-ATCGGCCCCAGCCAATG-3'), nSMase1 (right 5'- CCCGTCCACTCTTCTCAGTA-3' and left 5'- GTGCCGGGATCTCAAC AT-3'), nSMase2 (right 5'- TGAACATATTGAGCCCTTG C-3' and left 5'-CTTTGCCACACGACATTGC-3'), and \(\beta\)-actin (right 5'-TCAGGCCAG TCAATGCTCTTC-3' and left 5'-GCTTGAACCTTGGCAGAAGA-3').

2.8 Statistical analysis

Data are expressed as means \(\pm\) SEM; \(n\) indicates the number of animals, arteries, or cells tested. For multiple comparisons (e.g. the effects of various inhibitors against a control), statistical analysis was performed using a one-way ANOVA followed by a Bonferroni post hoc test, otherwise (e.g. control vs. single treatment) using a two-tailed Student's \(t\)-test for paired or unpaired observations. Differences were considered statistically significant when \(P < 0.05\).

3. Results

3.1 Effects of hypoxia on ceramide production in isolated pulmonary artery smooth muscle cells

Exposure to hypoxia induced an increase in ceramide content in freshly isolated PASMC (Figure 1A) which was significant after 1 min of perfusion with the hypoxic solution and remained elevated for at least 5 min. We analysed the
3.2 Role of protein kinase Cζ and nSMase in hypoxia-induced contraction in isolated pulmonary artery

Hypoxia induced a contractile response in isolated small pulmonary arteries. In the absence of other vasoconstrictor agent, this response was small in magnitude but rapid, sustained, and reproducible (Figure 2A) and could also be observed in endothelium-denuded arteries (not shown). The magnitude of this response (0.08 ± 0.01 mN, n = 10) is approximately 20% of the maximal response induced by the thromboxane A2 mimetic U46619. This hypoxic contraction was strongly inhibited by the PKCζ pseudosubstrate inhibitory peptide (PKCζ-Pi) (Figure 2B) suggesting a role for this kinase in HPV. GW4869 and the chemically unrelated nSMase inhibitor manumycin A also inhibited the hypoxic-induced contraction (Figure 2B). The effects of desipramine as aSMase inhibitor were not analysed because it is a known Ca2+ channel antagonist and inhibits agonist- and depolarization-induced contractions in vascular smooth muscle. However, D609, another inhibitor of aSMase, had no effect on hypoxic contraction (Figure 2B).

3.4 Effects of exogenous SMase

As expected, exogenous addition of SMase from Bacillus cereus, a homologue of mammalian nSMase, produced a marked increase in ceramide content in isolated PASMC (Figure 4A). Moreover, SMase mimicked the effects of hypoxia, i.e. it produced a sustained contractile response (Figure 4B) of a similar magnitude (0.10 ± 0.04 mN, n = 5) to the response induced by hypoxia. However, the time course of this contraction was slower than that induced by hypoxia. This response was also present in endothelium-denuded arteries (not shown). Exogenous addition of C6-ceramide also induced a contractile response (0.05 ± 0.01 mN, n = 32, Figure 4C). This response was inhibited by the PKCζ-Pi but not by the nSMase inhibitor GW4869. The inhibitory effect of the Rho kinase inhibitor Y27632 and the L-type calcium channel blocker nifedipine on ceramide-induced contraction did not reach statistical significance, but the effects of both drugs combined were highly significant (Figure 4D).

3.5 Hypoxic Kᵥ channel inhibition

As expected, we found that hypoxia inhibited Kᵥ currents (Figure 5) with a similar time course to the hypoxia-induced ceramide production and contraction. These effects, as shown above for hypoxia-induced vasoconstriction, were...
The mRNAs of aSMase and nSMase1 were similarly expressed in PA and mesenteric arteries as measured by real-time RT–PCR (Figure 6A). However, the mRNA expression of nSMase2 was much higher in PA when compared with mesenteric arteries. The protein expression of nSMase2, PKCζ, and Kv1.5 was also higher in PA as measured by western blot (Figure 6B). In addition, hypoxia failed to generate ceramide (Figure 6C) in mesenteric arteries. However, exogenous SMase induced a contractile response in isolated pulmonary artery mounted in a wire myograph exposed to SMase (B) and C₆-ceramide (C) at time 0 (representative tracings). (C) Effects of the nSMase inhibitor GW4869 (10 μmol/L, GW), protein kinase Cζ-pseudosubstrate inhibitory peptide (10 μmol/L), the L-type Ca²⁺ channel inhibitor nifedipine (1 μmol/L, Nifed), the Rho kinase inhibitor Y27632 (10 μmol/L), or the combination of Nifed + Y27632 on the contraction induced by C₆-ceramide (10 μmol/L) in PA. Results are means ± SEM of 6–10 experiments (except controls where n = 32). * and ** indicates P < 0.05 and 0.01, respectively, vs. control (ANOVA followed by a Bonferroni’s test).

3.6 Pulmonary selectivity

The mRNAs of aSMase and nSMase1 were similarly expressed in PA and mesenteric arteries as measured by real-time RT–PCR (Figure 6A). However, the mRNA expression of nSMase2 was much higher in PA when compared with mesenteric arteries. The protein expression of nSMase2, PKCζ, and Kv1.5 was also higher in PA as measured by western blot (Figure 6B). In addition, hypoxia failed to generate ceramide (Figure 6C) in mesenteric arteries. However, exogenous SMase induced a contractile response in isolated pulmonary arteries. The nSMase inhibitor GW4869 reverses hypoxic pulmonary vasoconstriction in vivo. Recordings of systemic (left panel, in closed chest rats via a catheter in the carotid artery) and pulmonary (right panel, in open chest rats via a catheter advanced through the right ventricle into the main pulmonary artery) arterial pressure in anesthetized ventilated rats treated with DMSO (vehicle) (A) or 1 mg Kg⁻¹ of GW4869 (B) administered intraperitoneally. Rats were exposed to alveolar hypoxia (10% O₂) as indicated.
mesenteric arteries which was similar to that induced in PA (Figure 6D).

4. Discussion

Herein we show that the activation of nSMase is required for acute HPV (Figure 7). The specific inhibitors of nSMase but not of aSMase strongly inhibited the constrictor response to hypoxia in small PA. The time course of ceramide generation induced by hypoxia in isolated PASMC and the mimicking effect of exogenous addition of SMase or ceramide are consistent with this view. These events were observed in non-genetically modified systems in vitro (rat PA or freshly isolated PASMC) and the role of nSMase was also confirmed in vivo by using the specific inhibitor GW4869. The hypoxia-induced increase in ceramide was not observed in mesenteric arteries. In addition, the expression of nSMase2 as well as other signalling proteins PKCζ and KV1.5 was higher in small PA vs. mesenteric arteries.

HPV in vivo is a rapid and sustained response. Because HPV responses are more readily and consistently observed when the small PAs are pre-constricted, the addition of a pre-constrictor agent has been a common practice in many studies analysing HPV in vitro. In pre-constricted rat PA, hypoxia produces a tri-phasic response, with an initial vasoconstriction followed by vasodilation and a late slow-developing contraction. However, under the right conditions of stretch, a sustained constrictor response to hypoxia has been reported with no added constrictor agent.21 In order to avoid a possible influence of a pre-construction agent on the intracellular signalling mediating HPV, in the present study hypoxic contractions were carried out in the absence of an agonist.

Several broad inhibitors of PKC isoforms have demonstrated to be effective in inhibiting HPV in isolated perfused lungs.26 PKCζ is activated by hypoxia in alveolar epithelial cells27 and is a known modulator of Kv currents in PA.13,14 Our data showing that PKCζ-Pi prevented the vasoconstriction induced by hypoxia strongly suggest that PKCζ is involved in the signalling pathway of HPV.

PKCζ is directly activated by ceramide.15 Herein, we show that hypoxia induced a rapid increase in ceramide content. The role of nSMase as a source of ceramide was confirmed using the specific inhibitor GW4869,23 whereas inhibition of aSMase with desipramine was without effect. GW4869 and the chemically unrelated nSMase inhibitor manumycin (but not the aSMase inhibitor D609) inhibited the vasoconstrictor...
nSMase in hypoxic pulmonary vasoconstriction

Figure 7 Diagram illustrating the proposed signalling cascade involved in hypoxic pulmonary vasoconstriction. The drugs used in the present work as well as their targets are also shown.

This specificity may be related in part to a higher expression of nSMase2 in small PA. However, it seems also possible that differences in the upstream mechanisms activating nSMase (e.g. the oxygen sensor or the redox response to hypoxia) may account for the pulmonary specificity. The expression of other signalling proteins involved, i.e. PKC_ζ and K_+1,5 (a specific K_v channel protein suggested to be involved in HPV^{11}), was also higher in PA compared with mesenteric arteries. However, exogenous addition of SMase also induced a contractile response in mesenteric arteries suggesting that pulmonary selectivity is related to differences in the activation of nSMase rather than in the response to SMase.

Generalized alveolar hypoxia associated with altitude, atelectasis, chronic obstructive pulmonary disease, or sleep apnea leads to HPV and, hence, pulmonary hypertension. The present study identifies nSMase and PKC_ζ as two novel mediators of acute HPV. It is noteworthy that nSMase does not appear to play a role in the control of systemic vascular tone as opposed to its downstream effectors K_v or L-type Ca^{2+} channels, suggesting that their inhibitors may selectively inhibit HPV without inducing systemic hypotension, a common side effect of vasodilators used in pulmonary hypertension. Interestingly, aSMase and PKC_ζ have been also proposed as therapeutic targets in acute lung injury^{25} and asthma,^{32} respectively.

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