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NS1619-induced vasodilation is enhanced and differentially mediated in chronically hypoxic lungs

Danielle J McCullough^{a,b}, Alexander Vang^a, and Gaurav Choudhary^{a,b}

^aVascular Research Laboratory, Providence VA Medical Center, Providence, RI, USA

^bDepartment of Medicine, Warren Alpert Medical School of Brown University, Providence, RI, USA

Abstract

Purpose: To identify the effect of the benzimidazole derivative, NS1619, on modulating pulmonary vascular tone in lungs from rats exposed to normoxia (21% F_iO₂) or chronic hypoxia (10% F_iO₂) for 3 weeks.

Methods: Isolated perfused lungs were precontracted (U46619) and dose-dependent vasodilation to NS1619 was assessed. To elucidate the mechanisms responsible, NS1619 vasodilatory responses were assessed following inhibition of large-conductance Ca²⁺-activated (BK_{Ca}; iberiotoxin and paxilline), L-type Ca²⁺ (nifedipine), K⁺ (tetraethylammonium), Cl⁻ (niflumic acid) and cation/TRP (lanthanum) channels, as well as nitric oxide synthase (L-NAME).

Results: Compared to normoxia, NS1619-induced vasodilation was significantly greater following hypoxia, however, NO-dependent vasodilation and BK_{Ca}-mediated vasodilation, in response to NS1619, was similar in the normoxic and hypoxic lungs. In contrast, direct activation of L-type Ca²⁺ and non-BK_{Ca} K⁺ channel was involved in the NS1619-induced vasodilation only in hypoxic lungs.

Conclusions: NS1619 causes pulmonary vasodilation by affecting multiple complementary pathways including stimulation of NO production, activation of BK_{Ca} channels, other TEA-sensitive K⁺ channels and L-type Ca²⁺ channels, and could be considered as a therapeutic agent in hypoxic PH.

Keywords

isolated perfused lung; NS1619; hypoxia; pulmonary hypertension; pulmonary vasodilation

INTRODUCTION

The development of pulmonary hypertension (PH), particularly in response to prolonged hypoxia, is of critical importance in patients suffering from chronic lung diseases and is associated with significant morbidity and mortality [1]. The underlying pathophysiology of

Corresponding Author: Gaurav Choudhary, M.D., Providence VA Medical Center, 830 Chalkstone Avenue, Providence, RI 02908, USA, gaurav_choudhary@brown.edu, Tel: 401-273-7100 ext. 2029, Fax: 401-457-3305.

Conflict of Interest: None

PH associated with chronic hypoxemia includes endothelial dysfunction, an enhanced vascular tone and vascular remodeling [2–4]. While the structural remodeling predominantly occurs in the large pulmonary arteries, the increase in pulmonary vascular resistance is largely attributed to pathological changes occurring within the pre-capillary arterioles. However, with the exception of supplemental oxygen therapy, there are currently no approved treatment strategies that effectively target the pulmonary resistance vasculature in PH associated lung disease.

In hypoxia-induced PH, changes in the activity and expression of the L-type Ca^{2+} channels [5], as well as voltage-gated (K_v) [6] and large conductance Ca^{2+} -activated potassium channels (BK_{Ca}) [7,8], have been observed and contribute, in part, to an elevated vascular tone and hypersensitivity to vasoconstrictor stimuli [9]. Previously we [10], and others [11,12], have shown that the benzimidazole derivative, NS1619, activates endothelial and vascular smooth muscle BK_{Ca} channels, and causes both endothelial dependent and independent vasodilation. Additionally, NS1619 has been reported to promote vasorelaxation through inhibitory actions on voltage-dependent Ca^{2+} channels [11,13]. Hence, NS1619 has the potential to favorably alter the activity of pulmonary vascular ion-channels that have been shown to be important in hypoxic lung vasculature. Therefore, the purpose of the current study was to determine the effect of NS1619 on pulmonary circulation in normoxic and chronically hypoxic animals using the isolated perfused lung technique, and to elucidate the underlying mechanisms responsible for the differences observed within hypoxic lung vasculature.

METHODS

Animals

All procedures were approved by the Animal Care and Use Committee at the Providence Veterans Affairs Medical Center and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Adult male Sprague–Dawley rats ($n = 85$, ~ 250 g; Charles River Laboratories, Wilmington, MA) were used in this study. Rats were housed at 23°C, maintained on a 12:12-hr light-dark cycle in either normoxic ($n = 38$; 21% FiO_2) or normobaric hypoxic ($n = 49$; 10% FiO_2 , Biospherix A chamber + Pro-Ox controller, Biospherix Ltd., Lacona, NY) environment for three weeks, and provided rat chow and water ad libitum.

Assessment of RV Hypertrophy

At the end of three weeks, the rats ($n = 7/\text{group}$) were anesthetized with isoflurane (2%/O₂ balance) and a transthoracic echocardiogram (Visualsonics Vevo 2100, FUJIFILM Visualsonics Inc., Toronto, Canada) was performed. The pulse-wave Doppler recording, at the right ventricular outflow tract, was used to measure pulmonary acceleration time to confirm development of pulmonary hypertension in the rats exposed to hypoxia, as previously reported [14].

Isolated ventilated perfused lungs

At the end of three weeks, the rats were anesthetized (sodium pentobarbital, 60mg/kg ip), injected with ip heparin, and the trachea was cannulated and connected to a volume-cycled ventilator. Catheters were placed into the pulmonary artery (via the right ventricle) and left atrium (via the left ventricle) and secured. The heart and lungs were then trimmed free from the thorax and diaphragm, removed *en bloc*, and suspended. The pulmonary vasculature was continuously perfused at 7 ml/min with Earle's balanced salt solution, maintained at 37 °C, and aerated with 95% O₂-5% CO₂ [15] and 21% O₂/5% CO₂/74% N₂ [16] for the normoxic and hypoxic lung preparations, respectively. Select experiments were repeated using 21% O₂/5% CO₂/74% N₂ aerated perfusate in normoxic lung preparations. A solution pH of 7.30–7.45 was maintained throughout the procedure.

Once suspended, the lungs were precontracted with a thromboxane mimetic, U46619, until a pressor response ($PAP_{U46619} - PAP_{Baseline}$) of ~10 mmHg was achieved and maintained. Peak PAP_{NS1619} was assessed following incremental doses of the putative BK_{Ca} channel activator, NS1619 (3/30/150 μM), added to the perfusate. In select experiments, to isolate the mechanism of the NS1619-induced responses in determining PAP_{NS1619} , lung preparations were perfused with iberiotoxin (IbTx; 200 nM; BK_{Ca} channel inhibitor [17]) paxilline (3 μM; BK_{Ca} channel inhibitor [18]), tetraethylammonium (TEA; 10 mM; non-selective K⁺ channel blocker [10]), L-NAME (100 μM; nitric oxide synthase inhibitor [10]), lanthanum (La³⁺; 100 μM; non-specific cation/TRP channel blocker [10]), niflumic acid (NFA; 30 μM; Cl⁻ channel inhibitor [19]), or nifedipine (1 μM; L-type Ca²⁺ channel blocker [19]) prior to the addition of NS1619.

The baseline PAP ($PAP_{Baseline}$) at was calculated as a mean pressure over forty seconds prior to the addition of U46619. Data is reported as percent precontraction, or percent vasorelaxation, and is determined by the following equations;

$$\begin{aligned} \% \text{ Precontraction} &= \left[(PAP_{NS1619} - PAP_{Baseline}) / (PAP_{U46619} - PAP_{Baseline}) \right] * 100 \\ \% \text{ Vasorelaxation} &= \left[(PAP_{U46619} - PAP_{NS1619}) / (PAP_{U46619} - PAP_{Baseline}) \right] * 100 \end{aligned}$$

All chemicals were obtained from Sigma Inc. (St. Louis, MO) with the exception of Ibtx, which was purchased from Anaspec (Fremont, CA).

Statistical Analysis

Dose-response curves were compared using a Student's *t* test, or repeated measures two-way ANOVA with Tukey's *post hoc* analyses for pairwise comparisons, as appropriate. Values are expressed as means ± SE. Statistical significance was defined as $P < 0.05$.

RESULTS

Animal characteristics

As expected, hypoxic rats weighed significantly less than their normoxic counterparts at the time of sacrifice (Normoxic: 401.3 ± 7.6g, Hypoxic: 354.1 ± 8.3g; $P < 0.05$). Heart rate (Normoxic: 376 ± 15 bpm vs. Hypoxic: 363 ± 11 bpm; $P = NS$) and LV ejection fraction

(Normoxic: $87.6 \pm 1.7\%$ vs. Hypoxic: $86.5 \pm 2.8\%$; $P = \text{NS}$) were not significantly different between rats exposed to normoxia or hypoxia. However, RV thickness by echocardiogram (Normoxic: 0.61 ± 0.06 mm vs. Hypoxic: 1.5 ± 0.06 mm; $P < 0.05$; $n = 7$), and RV/LV weight ratio (Normoxic: 0.53 ± 0.02 vs. Hypoxic: 0.87 ± 0.1 ; $P < 0.05$; $n = 4$) was significantly higher, and pulmonary acceleration time (Normoxic: 39.8 ± 1.5 msec vs. Hypoxic: 25.9 ± 2.3 msec; $P < 0.05$; $n = 7$) significantly lower, in hypoxic animals, validating the efficacy of the PH protocol.

NS1619-induced vasodilatory response in normoxic and hypoxic lungs

The selective BK_{Ca} channel opener NS1619 elicited a dose-dependent vasodilation in the U46619-precontracted lungs from normoxic and hypoxic rats, however, NS1619 mediated vasodilation was significantly greater (~20% more) in lungs from hypoxic animals compared to normoxic (Fig. 1a & b). Additionally, a subset ($n=4$) of lungs perfused with 21% $\text{O}_2/5\%$ CO_2 were investigated to assess the potential influence of variable perfusate O_2 concentrations as a confounding factor, however the NS1619 mediated vasodilation remained significantly less compared to that observed in hypoxic lungs ($63.8 \pm 3.4\%$ of maximal vasoconstriction with $30\mu\text{M}$ NS1619). Vasodilatory responses to the exogenous nitric oxide donor, sodium nitroprusside (SNP; 10^{-9} - 10^{-4} M), elicited dose-dependent vasodilation within both groups, but there was no significant difference in SNP-induced dilation between groups (Fig. 1c), confirming vasodilatory capacity was not affected by hypoxic conditions.

Differential effect of ion channel blockers on NS1619 mediated vasodilation in hypoxic lungs

The mechanism underlying the vasodilatory effect of BK_{Ca} channel activation is membrane hyperpolarization, and the concomitant inhibition of L-type Ca^{2+} channels, in vascular smooth muscle cells [20]. Hence, we tested the effect of BK channel blocker, Ibtx, and L-type Ca^{2+} channel blocker, nifedipine, on NS1619-induced vasodilation. Pretreatment with Ibtx and nifedipine significantly attenuated NS1619-induced vasodilation in lung preparations from normoxic rats to a similar extent (Fig. 2a & b). However, although Ibtx significantly attenuated the vasodilation in lungs from hypoxic animals (Fig. 2c & d), the inhibition was significantly less compared to the effect of nifedipine (Fig. 2c & d), suggesting that NS1619 causes vasodilation in hypoxic lungs, in part, by a non- BK_{Ca} mechanism. Also, pretreatment of the lungs with paxilline, a structurally unrelated BK blocker, resulted in attenuation of vasodilation similar to that by Ibtx (Normoxia: $64.7 \pm 4.1\%$ vs. $76.0 \pm 5.3\%$, Hypoxia: $42.2 \pm 6.2\%$ vs. $50.2 \pm 7.6\%$, respectively).

In order to assess contribution of alternate potassium channels, we next compared the effect of Ibtx with a non-specific potassium channel blocker, TEA, on NS1619-induced vasodilation. Both TEA and Ibtx caused similar attenuation of the NS1619-induced vasodilation in normoxic lung (Fig. 3a & b). In contrast, TEA caused significantly more inhibition of NS1619-induced vasodilation in hypoxic lungs compared to Ibtx (Fig. 3a & b), suggesting a role of non- BK_{Ca} K^+ channels in mediating the NS1619-induced vasodilation.

To determine if Cl^- channel activation may underlie the effect of NS1619 in addition to K^+ channels, we pretreated the lungs with Cl^- channel blocker, NFA, in addition to TEA and observed no additional attenuation of the vasodilation with TEA alone (Normoxia: $68.9 \pm 5.8\%$ vs. $83.9 \pm 7.0\%$, Hypoxia: $60.3 \pm 5.3\%$ vs. $68.2 \pm 5.8\%$, respectively).

NO-dependent effects of NS1619

Since activation of BK_{Ca} channels is associated with nitric oxide (NO) production in endothelial cells (related to an increase in intracellular Ca^{2+} [10], we next assessed the NO-dependent vasodilation in normoxic and hypoxic lungs. In both normoxic and hypoxic lungs, inhibition of NO production (L-NAME; Fig. 4a) significantly reduced NS1619-induced vasodilation, compared to vehicle controls. However, the magnitude of reduction in both normoxic and hypoxic lungs was similar (Fig. 4b). We have previously shown that NS1619 activates BK_{Ca} channels in endothelial cells and causes NO release dependent on La^{3+} sensitive Ca^{2+} entry pathways [21]. Pretreatment of lungs with La^{3+} resulted in attenuation of NS1619-induced vasodilation (Fig. 4c) similar to that of L-NAME (Fig. 4d).

DISCUSSION

While vascular remodeling is a critical component of PH associated with hypoxia [3,4] there is evidence to support that tonic vasoconstriction plays a significant role in the high pulmonary vascular resistance characteristic of PH [22]. Although vascular ion channels play an important role in vasoregulation of the pulmonary circulation [9], there is significant heterogeneity in the distribution of ion channels between the macro- and microvasculature throughout the pulmonary circulation [23]. Furthermore, the expression and activity of several of these vascular ion-channels is altered in chronically hypoxic lungs [9,5,8]. Hence, using an isolated perfused lung preparation we sought to determine the effect of the benzimidazole derivative, NS1619, on the vasoreactivity of the precontracted pulmonary circulation and to identify the relative contribution of specific vascular ion channels in mediating the response in both normoxic and chronically hypoxic lungs. Our results demonstrate that the NS1619 causes significantly higher vasodilatory response in lungs from hypoxic animals. Furthermore, we show that the NS1619-induced vasodilation exhibits both NO-dependent and NO-independent components, and is primarily mediated via effects on vascular K^+ and L-type Ca^{2+} channels. Current therapies in pulmonary hypertension target vascular L-type calcium channels, endothelin pathway or nitric oxide pathway. Our observations suggest that another approach may be to study small molecules such as NS1619 that favorably affect pulmonary vascular function by targeting multiple pathophysiologically relevant pathways. Further studies in preclinical models are needed to confirm this approach and compare it to other available therapies.

BK_{Ca} channels present in the pulmonary vascular endothelial (PVEC) and smooth muscle cells (PVSMC) open in response to depolarization and elevated intracellular calcium ($[\text{Ca}^{2+}]_i$) levels, resulting in membrane hyperpolarization [20]. In endothelial cells, the hyperpolarization may result in a further increase in $[\text{Ca}^{2+}]_i$ resulting in release of vasodilatory mediators such as NO [24], whilst within vascular smooth muscle cells, the hyperpolarization can promote vasodilation by decreasing the activity of voltage-dependent

L-type Ca^{2+} channels [25]. In chronic hypoxia, BK_{Ca} channel activation has been shown to underlie the vasodilatory and anti-mitogenic effects of carbon monoxide inhalation [26] and dehydroepiandrosterone (DHEA) administration [27], respectively. Correspondingly, we found that activation of BK_{Ca} channels resulted in vasodilation of the pulmonary vasculature from both normoxic and hypoxic animals (~ 29% in normoxia and 25% in hypoxic lungs). Although it has been demonstrated that chronic hypoxia promotes an increased expression of BK_{Ca} channels in rat PVSMC [8], in the current study the activation of the channels with NS1619 did not show an increased BK_{Ca} mediated vasodilation in the hypoxic lungs. This apparent discrepancy may be related to the observation that the BK_{Ca} channels already have a higher basal activity in settings of hypoxia, which is considered to be a compensatory mechanism associated with increased $\beta 1$ -subunit expression [8] and increased intracellular calcium levels in PVSMC [28].

Nitric oxide plays an important role in pulmonary vascular reactivity and is considered a therapeutic target in pulmonary hypertension [29]. Previously, we have demonstrated a NS1619-induced hyperpolarization of PVEC and increased NO production *in vivo* [10], and that the hyperpolarization is attenuated by blocking non-specific cation channels *in vitro* [21]. Consistent with the mechanism we previously observed in cell culture [21], pretreatment with L-NAME (NOS inhibitor) and La^{3+} (TRP inhibitor) attenuated the NS1619-induced vasodilation, suggesting that the NS1619-induced vasodilation is partly endothelial dependent and related to an increase in extracellular Ca^{2+} entry within the endothelium. However, in the current study, there was no apparent difference in the endothelial dependent vasodilation between the normoxic and hypoxic lungs. Prior studies have demonstrated that eNOS expression and activity is augmented in hypoxic lungs and results in enhanced endothelial dependent vasodilation in response to certain vasodilators, such as histamine[30]. In contrast, exogenous NO donors show an impaired vasodilatory response in hypoxic lungs likely due to endothelium derived ROS and ET-1 counteracting the actions of exogenous NO in hypoxic lungs [31]. These studies suggest that in order to improve endothelium dependent vasodilation, a simultaneous increase in NO release and inhibition of endothelial ROS and ET-1 may be required. In the current study, we did not observe an enhanced NO dependent vasodilation in hypoxic lungs in response to NS1619. It is possible that although NS1619 increases NO release in hypoxic vasculature, it may not directly affect ROS and/or ET-1 release similar to other endothelial dependent vasodilators, such as histamine.

Thus, we found that the extent of NO-dependent vasodilation and BK_{Ca} mediated vasodilation, in response to NS1619, was similar in the normoxic and hypoxic lungs. However, pretreatment with L-type Ca^{2+} channel blocker, nifedipine, had a larger inhibitory effect in hypoxic lungs than in normoxic lungs. This would be expected as a result of NS1619 inhibiting either L-type Ca^{2+} channels directly or by activating other K^{+} channels in VSMC resulting in hyperpolarization and indirect inhibition of L-type Ca^{2+} channels. We find that pretreatment with TEA, a BK_{Ca} and non-specific K^{+} channel blocker, had similar effect as nifedipine in hypoxic lungs and caused significantly more inhibition than Ibtx alone. Hence, it would appear the higher NS1619-induced vasodilation in hypoxia could be a result of activation of other TEA-sensitive PVSMC K^{+} channels involved in the hypoxic pulmonary vasoconstrictor response, such as $\text{K}_{\text{v}} 2.1$ [32,33].

The results from our study have to be interpreted in context of certain limitations. Since, we did not simultaneously block both L-type Ca^{2+} channels and K^{+} channels, it is possible that some effect of NS1619 is mediated by direct inhibition of L-type Ca^{2+} channels that have been shown to be upregulated in hypoxic lungs as well [5]. Also, we chose to use similar pressor response instead of similar U46619 doses as previously used by others [30] in our precontraction protocol. Hence, we cannot completely rule out that use of varying doses of U46619 could have had an effect on subsequent dose-response curves. Although we performed select experiments with 21% O_2 perfusate conditions to demonstrate that NS1619 response remained significantly higher in chronically hypoxic lungs as with 95% O_2 perfusate conditions, we can not rule out the effect of 21% O_2 perfusate in chronically hypoxic lungs on the dose responsiveness of NS1619 in presence of other inhibitors.

In conclusion, we demonstrate that NS1619 causes robust vasodilation in the pulmonary circulation that is further accentuated in lungs from hypoxic animals. Also, NS1619 mediated the vasodilation by affecting multiple complementary pathways including stimulation of NO production, activation of BK_{Ca} channels, other TEA-sensitive K^{+} channels and L-type Ca^{2+} channels. Hence, compounds like NS1619 represent a novel therapeutic strategy of targeting multiple channels in hypoxic pulmonary hypertension and could be considered for further preclinical evaluation.

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Fig. 1.

Concentration-response relation (a) of isolated perfused lungs from normoxic and hypoxic rats ($n = 6/\text{group}$) to the BK_{Ca} channel activator, NS1619 (vehicle). Graphical representation (b) of the resultant percent vasorelaxation in response to NS1619 (30 μM) in normoxic and hypoxic lungs. Concentration-response relation (c) of isolated perfused lungs from normoxic and hypoxic rats ($n = 4/\text{group}$) to the exogenous NO-donor, sodium nitroprusside (SNP). Values are means $\pm$ SE. * $P < 0.05$ vs. normoxia

Fig. 2.

Respective concentration-response relation and resultant percent vasorelaxation ([NS1619]=30 μM) of isolated perfused lungs from normoxic (a & b) and hypoxic (c & d) rats ($n = 3-6/\text{group}$) to the BK_{Ca} channel activator, NS1619, in the absence (vehicle) and presence of iberiotoxin (Ibtx; 200 nM; membrane BK_{Ca} channel inhibitor) and nifedipine (1 μM ; L-type Ca^{2+} channel blocker). Values are means $\pm$ SE. * $P < 0.05$ vs. vehicle. # $P < 0.05$ vs. Ibtx

Fig. 3. Concentration-response relation (a) and graphical representation of the resultant percent vasorelaxation (b; [NS1619]= 30 μM) of isolated perfused lungs from normoxic and hypoxic rats ($n = 3-7$) to the BK_{Ca} channel activator, NS1619, in presence of iberiotoxin (Ibtx; 200 nM; selective BK_{Ca} channel inhibitor) and tetraethylammonium (TEA; 10 mM; non-specific K^+ channel blocker). Values are means $\pm$ SE. * $P < 0.05$ vs. Ibtx within same condition

Fig. 4.

Concentration-response relation of isolated perfused lungs from normoxic and hypoxic rats ($n = 4-6/\text{group}$) to NS1619 in the absence (vehicle) and presence of (a) L-NAME (100 μM ; nitric oxide synthase inhibitor) and (c) lanthanum (La^{3+} ; 100 μM ; non-specific cation/TRP channel blocker). Qualitative representation of the relative contribution of NO-dependent and NO-independent mechanisms to vasorelaxation (b). Comparison of the resultant percent vasorelaxation, in response to NS1619 (30 μM), of normoxic and hypoxic lungs in the presence of L-NAME and La^{3+} (d). Values are means $\pm$ SE. * $P < 0.05$ vs. vehicle within same condition