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Direct effect of chronic hypoxia in suppressing large conductance Ca^{2+}-activated K^+ channel activity in ovine uterine arteries via increasing oxidative stress

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Key points

- Chronic hypoxia has a direct effect in down-regulating the BK_{Ca} channel β1 subunit and inhibiting the BK_{Ca} channel activity in uterine arteries of pregnant sheep.
- Oxidative stress plays a causal role in hypoxia-mediated suppression of BK_{Ca} channel function.
- The steroid hormone-induced effect on BK_{Ca} channels is a target of hypoxia-mediated oxidative stress.
- Inhibition of oxidative stress ameliorates the adverse effect of hypoxia both *ex vivo* and *in vivo* in pregnant sheep exposed to long-term high-altitude hypoxia.
- Our findings provide novel evidence of a causative role of oxidative stress in hypoxia-mediated inhibition of the BK_{Ca} channel activity in uterine arteries and new insights in understanding and alleviating pregnancy complications associated with gestational hypoxia such as pre-eclampsia and fetal growth restriction.

Abstract Uterine arteries of pregnant sheep acclimatized to long-term high-altitude hypoxia were associated with a decrease in large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activity. The present study tested the hypothesis that prolonged hypoxia has a direct effect in suppressing BK_{Ca} channel activity by increasing oxidative stress. Uterine arteries were isolated from non-pregnant and near-term (~142 days) pregnant sheep, and were treated *ex vivo* with 21.0 or 10.5% O_2 for 48 h. The hypoxia treatment significantly increased the production of reactive oxygen species in uterine arteries, which was blocked by *N*-acetylcysteine. In uterine arteries of pregnant sheep, hypoxia significantly inhibited BK_{Ca} channel current density, decreased NS1619-induced relaxations and increased pressure-dependent tone, which were annulled by *N*-acetylcysteine. In accordance, hypoxia resulted in down-regulation of BK_{Ca} channel β1 subunit, which was restored in the presence of *N*-acetylcysteine. In addition, the *N*-acetylcysteine treatment significantly increased BK_{Ca} channel β1 subunit abundance and BK_{Ca} channel current density in uterine arteries from pregnant sheep exposed to high-altitude hypoxia (3801 m, P_{aO_2}: 60 mmHg) for 110 days. In uterine arteries of non-pregnant animals, hypoxia inhibited steroid hormone-induced up-regulation of BK_{Ca} channel current density and NS1619-mediated relaxations, which were reversed by *N*-acetylcysteine. Furthermore, the synthetic superoxide dismutase and catalase mimetic EUK-134 also ablated the effects of hypoxia on BK_{Ca} channel currents in uterine arteries. The results demonstrate a direct effect of hypoxia in inhibiting the BK_{Ca} channel activity in uterine arteries via increased oxidative stress.

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Abbreviations BK_{Ca}, large conductance Ca^{2+}-activated K^+; DCF, 2′,7′-dichlorodihydrofluorescein; DCFH-DiOxyQ, dichlorodihydrofluorescin DiOxyQ; DHE, dihydroethidium; DMEM, Dulbecco's Modified Eagle's medium; ER-α, oestrogen receptor alpha; ER-β, oestrogen receptor beta; Nox, NADPH oxidase; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+; KCNMB 1, Ca^{2+}-activated K^+ subunit beta-1; $ONOO^-$, peroxynitrite anion; P_{aO_2}, partial pressure of arterial oxygen; PKC, protein kinase C; PSS, physiological saline solution; RNS, reactive nitrogen species; ROO, peroxyl radical; ROS, reactive oxygen species; SK_{Ca}, small conductance Ca^{2+}-activated K^+; SOD, superoxide dismutase; VSMCs, vascular smooth muscle cells.

Introduction

Marked changes in the cardiovascular system occur during normal pregnancy to accommodate fetal growth; one of major haemodynamic adaptions is the dramatic increase in uterine blood flow (Rosenfeld, 1977; Palmer *et al.* 1992). Adequate uterine blood supply during pregnancy is essential for the development/growth of the placenta and fetus, as well as the well-being of the mother. Aberrant uterine perfusion is associated with pregnancy complications such as pre-eclampsia and eclampsia (Lang *et al.* 2003; Browne *et al.* 2015). These complications are often associated with significant maternal morbidity and mortality and fetal growth restriction (Lambert *et al.* 2014). Gestational hypoxia is a notorious insult to maternal cardiovascular homeostasis and increases the incidence of pre-eclampsia and fetal growth restriction by impairing the uteroplacental circulation (Zamudio *et al.* 1995*a*,*b*; Palmer *et al.* 1999). The large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel plays a pivotal role in regulating the membrane potential of vascular smooth muscle cells (VSMCs) and thus vascular tone (Hill *et al.* 2010; Hu & Zhang, 2012). The channel opening, predominantly stimulated by an increase in intracellular Ca^{2+} concentrations, results in membrane hyperpolarization and reduces the activity of voltage-dependent Ca^{2+} channels. Increased expression of the BK_{Ca} channel β1 subunit and enhanced channel activity in uterine arteries during pregnancy attribute to decreased uterine vascular tone and increased uterine blood flow (Rosenfeld *et al.* 2001, 2009; Hu *et al.* 2011). Oestrogen has been found to play a critical role in the upregulation of the BK_{Ca} channel β1 subunit and reduced pressure-dependent tone in uterine arteries during pregnancy (Nagar *et al.* 2005; Hu *et al.* 2011; Chen *et al.* 2014). Our recent studies demonstrated that long-term high-altitude hypoxia during gestation in sheep was associated with a decrease in the BK_{Ca} channel activity, leading to an increase in pressure-dependent myogenic tone in uterine arteries (Hu *et al.* 2012; Zhu *et al.* 2014). Furthermore, chronic hypoxia nullified steroid hormone-mediated upregulation of BK_{Ca} channel activity and increased pressure-dependent tone of uterine arteries (Hu *et al.* 2012; Zhu *et al.* 2014).

Whereas these initial observations of decreased BK_{Ca} channel activity in uterine arteries *of* pregnant sheep acclimatized to long-term high-altitude hypoxia were of high interest, the question arose is to what extent this effect observed in the *in vivo* studies was a direct effect of hypoxia on uterine arteries or was a response to indirect mediators resulting from the hypoxic stress in animals. In addition, the mechanisms underlying the hypoxia-mediated inhibition of BK_{Ca} channel activity in uterine arteries remain largely elusive. Hypoxia has been shown to promote production of reactive oxygen species (ROS) and to increase oxidative stress in the cardiovascular system (Giordano, 2005; Fresquet *et al.* 2006; Liu *et al.* 2006). The excessive production of ROS and increased oxidative stress in the placenta and vasculature have been observed in pregnancy complications including pre-eclampsia (Raijmakers *et al.* 2004; Siddiqui *et al.* 2010). The NADPH oxidase (Nox) family is of critical importance in the regulation of ROS production (Drummond *et al.* 2011). Our recent studies demonstrated that long-term high-altitude hypoxia during gestation increased ROS production in ovine uterine arteries, in part due to increased expression and activity of Nox2 (Xiao *et al.* 2013). It has been shown that ROS are implicated in down-regulating BK_{Ca} channels, as well as small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+}-activated K^+ channels in the cardiovascular system (Lu *et al.* 2012; Zhao *et al.* 2014; Yi *et al.* 2015). Herein, we present novel evidence that prolonged hypoxia has a direct effect in inhibiting the BK_{Ca} channel activity in uterine arteries by increasing oxidative stress.

Methods

Ethical approval

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After tissue collection, animals were killed via intravenous injection of 15 ml T-61

solution (Hoechst-Rousel, Somerville, NJ, USA), according to American Veterinary Medical Association guidelines.

Tissue preparation and treatment

Uterine arteries were harvested from non-pregnant and near-term (~142–145 days of gestation) pregnant sheep (*Ovis aries*) maintained at ~300 m above sea level (low altitude) or exposed to high-altitude (3801 m) hypoxia (P_{aO_2}: 60 mmHg) for 110 days (starting from 30 days of gestation for pregnant animals) (Chang *et al.* 2010). Animals were anaesthetized via IV injection of propofol (2 mg kg^{-1}) followed by intubation, and anaesthesia was maintained on 1.5–3.0% isoflurane balanced in O_2 throughout the surgery. An incision was made in the abdomen and the uterus was exposed. The fourth generation branches of main uterine arteries and resistance-sized uterine arteries were isolated and removed without stretching and placed into a Krebs solution containing (in mM) 130.0 NaCl, 10.0 Hepes, 6.0 glucose, 4.0 KCl, 4.0 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.18 KH_2PO_4 and 0.025 EDTA (pH 7.4). To determine the direct effect of hypoxia, uterine arteries were treated *ex vivo* under normoxic or hypoxic conditions for 48 h. Given an ~50% decrease in arterial P_{aO_2} observed in high-altitude hypoxic sheep, uterine arteries were placed in a culture dish containing 5 ml of phenol red-free Dulbecco's Modified Eagle's medium (DMEM) supplemented with 1% charcoal-stripped fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and incubated at 37°C in humidified incubators for 48 h with oxygen levels at either 21.0% O_2 for normoxic or 10.5% O_2 for hypoxic conditions, as described previously (Chang *et al.* 2010; Dasgupta *et al.* 2012; Hu *et al.* 2012). For the hormonal treatment, uterine arteries from non-pregnant sheep were incubated in phenol red-free DMEM with 1% charcoal-stripped fetal bovine serum for 48 h at 37°C in a humidified CO_2 incubator under 21.0% O_2 and 10.5% O_2 in the absence or presence of 17β-estradiol (0.3 nM; Sigma, St Louis, MO, USA) and progesterone (100.0 nM; Sigma), as reported previously (Xiao *et al.* 2009; Chang *et al.* 2010; Hu *et al.* 2011). Phases of the ovarian cycle and systemic blood steroid levels were not determined in non-pregnant animals in the present study. The potential pre-exposure to 17β-estradiol and/or progesterone of uterine arteries from non-pregnant animals and their priming effects on the vessels could not be excluded. Each experimental group had four to five animals.

Relaxation studies

Uterine arteries were separated from surrounding tissues and cut into 2 mm ring segments. Isometric tension was measured in the Krebs solution in a tissue bath system (Radnoti, Monrovia, CA, USA) at 37°C as described previously (Chang *et al.* 2010; Xiao *et al.* 2013). Briefly, each ring segment was equilibrated for 60 min and then gradually stretched to the optimal resting tension determined by responses to three 120 mM KCl challenges. Tissues were then pre-contracted with submaximal concentrations of noradrenaline that produced ~70–80% of the maximal contraction, followed by additions of the BK_{Ca} channel opener NS1619 in a cumulative manner.

Measurement of pressure-dependent tone

Pressure-dependent tone of resistance-sized uterine arteries was measured as described previously (Chang *et al.* 2010; Hu *et al.* 2011). Briefly, the arterial segments (diameter ~150 μm) were mounted and pressurized in an organ chamber (Living Systems Instruments, Burlington, VT, USA). The intraluminal pressure was controlled by a servo-system to set transmural pressures, and arterial diameter was recorded using the SoftEdge Acquisition Subsystem (IonOptix LLC, Milton, MA, USA). After the equilibration period, the intraluminal pressure was increased in a stepwise manner from 10 to 100 mmHg in 10 mmHg increments, and each pressure was maintained for 5 min to allow vessel diameter to stabilize before the measurement. The passive pressure–diameter relationship was determined in Ca^{2+}-free physiological saline solution (PSS) containing 3.0 mM EGTA to determine the maximum passive diameter. The following formula was used to calculate the percentage of pressure-dependent tone at each pressure step: % tone = (D1 − D2)/D1 × 100, where D1 is the passive diameter in Ca^{2+}-free PSS (0 Ca^{2+} with 3.0 mM EGTA), and D2 is the active diameter with normal PSS in the presence of extracellular Ca^{2+}.

Measurement of ROS/RNS productions

Total ROS/reactive nitrogen species (RNS) production in uterine arteries was measured with the Oxiselect *in vitro* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer's instruction, as described previously (Patterson *et al.* 2012; Xiong *et al.* 2012; Xiao *et al.* 2013). The principle of this assay is that dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ) reacts with ROS/RNS such as hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), nitric oxide (NO) and peroxynitrite anion ($ONOO^-$), and the complex is rapidly oxidized to the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) in the cytosol. The intensity of DCF fluorescence is proportional to the amount of ROS/RNS in the biological specimen. Briefly, tissues were homogenized in PBS followed by centrifugation at 4°C for 10 mins at 10,000 *g*, and the supernatants were collected for ROS/RNS assay. A given amount of sample was incubated with the probe for ~30 min, and the fluorescence was determined with a

fluorescence plate reader at 480 nm excitation/530 nm emission. It appears that all techniques currently available to measure ROS/RNS have their own drawbacks (Dikalov *et al.* 2007; Chen *et al.* 2010). DCF may trigger an increase in ROS itself (Dikalov *et al.* 2007). Thus, ROS contents measured in the present study might be somewhat overestimated in tissues exposed to both normoxic and hypoxic environments. However, the DCF assay is still commonly used to measure ROS (Chen *et al.* 2010). Our previous studies revealed that assays using DCF and dihydroethidium (DHE) yielded comparable measurements in cells and tissues of various animal sources (Patterson *et al.* 2012; Xiong *et al.* 2012; Xiao *et al.* 2013).

Western immunoblotting

Protein abundance of the BK_{Ca} channel β1 subunit in uterine arteries was measured as described previously (Hu *et al.* 2011, 2012). Briefly, tissues were homogenized in lysis buffer followed by centrifugation at 4°C for 10 min at 10,000 *g*, and the supernatants were collected. Samples with equal proteins were loaded onto 7.5% polyacrylamide gel with 0.1% SDS and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes. After blocking non-specific binding sites by dry milk, membranes were incubated with primary antibodies against the BK_{Ca} channel β1 subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, membranes were incubated with secondary HRP-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software (Kodak, Rochester, NY, USA). The target protein abundance was normalized to the abundance of β-actin as a protein loading control.

Measurement of BK_{Ca} channel current

Arterial smooth muscle cells were enzymatically dissociated from resistance-sized uterine arteries, and whole-cell K^+ currents were recorded using an EPC 10 patch-clamp amplifier with Patchmaster software (HEKA, Lambrecht/Pfalz, Germany) at room temperature as previously described (Hu *et al.* 2011, 2012). Briefly, cell suspension drops were placed in a recording chamber, and adherent cells were continuously superfused with Hepes-buffered PSS containing (in mM) 140.0 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10.0 Hepes and 10.0 glucose (pH 7.4). Only relaxed and spindle-shaped myocytes were used for recording. Micropipettes were pulled from borosilicate glass and had resistances of 2–5 mΩ when filled with the pipette solution containing (in mM) 140.0 KCl, 1.0 $MgCl_2$, 5.0 Na_2ATP, 5.0 EGTA and 10.0 Hepes (pH 7.2). $CaCl_2$ was added to bring free Ca^{2+} concentrations to 100 nM as determined using WinMAXC software (Chris Patton, Stanford University). Cells were held at –50 mV, and whole-cell K^+ currents were evoked by voltage steps from −60 to +80 mV by stepwise 10 mV depolarizing pulses (350–ms duration, 10 s intervals) in the absence and presence of 1 mM BK_{Ca} channel blocker tetraethylammonium (Hu *et al.* 2011, 2012). BK_{Ca} currents, determined as the difference between whole-cell K^+ currents in the absence of tetraethylammonium and that in the presence of tetraethylammonium, were normalized to cell capacitance and expressed as picoamps per picofarad (pA pF^{-1}).

Pharmacological tools

To ascertain roles of ROS in the aberrant regulation of BK_{Ca} channel functions in uterine arteries, pharmacological tools *N*-acetylcysteine and EUK 134 were used in the present study. *N*-Acetylcysteine is a precursor of glutathione and functions as a free radical scavenger (Zafarullah *et al.* 2003; Sun, 2010), whereas EUK 134 is a superoxide dismutase (SOD) and catalase mimetic (Rong *et al.* 1999). Both compounds have been extensively used as tools to probe the role of ROS in physiological and pathophysiological processes (Zafarullah *et al.* 2003; Ajith & Jayakumar, 2014). Our previous studies demonstrated that *N*-acetylcysteine at 1 mM effectively ablated hypoxia- and noradrenaline-stimulated ROS generation in H9c2 cells and intact fetal hearts (Patterson *et al.* 2012; Xiong *et al.* 2012). Moreover, we demonstrated that *N*-acetylcysteine at 1 mM inhibited ROS-mediated suppression of BK_{Ca} channel activity in uterine arteries of pregnant sheep acclimatized to long-term high-altitude hypoxia (Zhu *et al.* 2014). Therefore, the same concentration of *N*-acetylcysteine was used in the present study. In addition, the functional roles of BK_{Ca} channels was probed using the BK_{Ca} channel opener NS1619 (Olesen *et al.* 1994; Holland *et al.* 1996). The specificity of NS1619 on BK_{Ca} channels in uterine arteries has been demonstrated previously using iberitoxin (Zhu *et al.* 2013).

Statistics

Data are expressed as means ± SEM obtained from the number of experimental animals given. Concentration–response curves were analysed by computer-assisted non-linear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences were evaluated for statistical significance ($P < 0.05$) by ANOVA or *t* test where

appropriate; and the concentration–response relationship was analysed with repeated measures ANOVA.

Results

Hypoxia increased ROS/RNS production in uterine arteries

Uterine arteries isolated from pregnant sheep were treated under normoxia (21.0% O_2) or hypoxia (10.5% O_2) for 48 h in the absence or presence of *N*-acetylcysteine. As shown in Fig. 1, the prolonged hypoxic treatment significantly increased ROS/RNS production in uterine arteries in the absence of *N*-acetylcysteine. *N*-Acetylcysteine had no significant effect on ROS/RNS production under normoxic conditions, but it ablated the hypoxia-induced increase in ROS/RNS production in uterine arteries (Fig. 1).

Antioxidants blocked hypoxia-mediated inhibition of BK_{Ca} channel current density in uterine arteries

BK_{Ca} channel currents were recorded in myocytes isolated from uterine arteries of pregnant sheep, treated with normoxia (21.0% O_2) or hypoxia (10.5% O_2) for 48 h in the absence or presence of antioxidants. As illustrated in Fig. 2*A*, hypoxia markedly inhibited BK_{Ca} channel current density (10.3 ± 0.8 *vs.* 28.0 ± 3.5 pA pF^{-1} at +80 mV; $P < 0.05$) in the absence of antioxidants. BK_{Ca} channel currents were not significantly altered by antioxidants in uterine arterial myocytes under normoxic conditions. However, *N*-acetylcysteine reduced the effect of hypoxia and partially restored the BK_{Ca} channel current density from 8.2 ± 0.9 to 19.3 ± 0.9 pA pF^{-1} at +80 mV ($P < 0.05$, Fig. 2*B*). Moreover, EUK 134 also ablated hypoxia-induced suppression of BK_{Ca} channel and the BK_{Ca} channel current density at +80 mV was elevated from 12.3 ± 1.6 to 29.4 ± 3.3 pA pF^{-1} ($P < 0.05$, Fig. 2*C*).

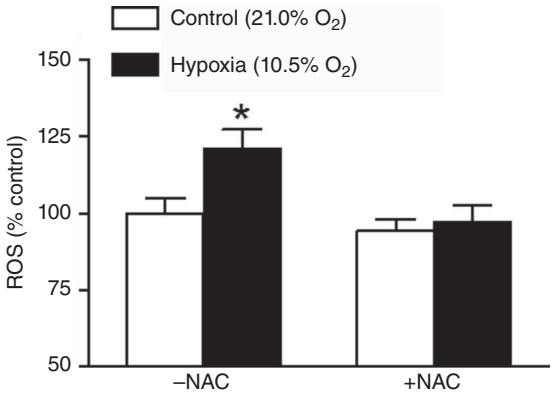

Figure 1. Hypoxia increased ROS in uterine arteries
Uterine arteries of pregnant sheep were treated under control (21.0% O_2) and hypoxia (10.5% O_2) for 48 h in the absence or presence of 1 mM *N*-acetylcysteine (NAC). ROS were measured with a DCF-based quantitative assay kit. Data are means ± SEM of four tissues from four animals of each group. Data were analysed with two-way ANOVA. *$P < 0.05$, hypoxia *vs.* control.

N-Acetylcysteine ablated hypoxia-mediated decrease in NS1619-induced relaxations in uterine arteries

Uterine arteries isolated from pregnant sheep were treated under normoxia (21.0% O_2) or hypoxia (10.5% O_2) for 48 h in the absence or presence of *N*-acetylcysteine. The BK_{Ca} channel opener NS1619 produced concentration-dependent relaxations of uterine arteries (Fig. 3). In the absence of *N*-acetylcysteine, the hypoxic treatment significantly decreased NS1619-induced relaxations (Fig. 3*A*). In the presence of *N*-acetylcysteine, the hypoxic effect was abrogated and there were no significant differences in NS1619-induced relaxations between normoxic and hypoxic conditions (Fig. 3*B*).

N-Acetylcysteine inhibited hypoxia-mediated increase in pressure-dependent tone in uterine arteries

BK_{Ca} channel activity plays a critical role in determining pressure-dependent tone in uterine arteries (Hu *et al.* 2011, 2012). We next determined whether the inhibition of ROS by *N*-acetylcysteine affected uterine arterial pressure-dependent response. As shown in Fig. 4*A*, in the absence of *N*-acetylcysteine, the hypoxic treatment resulted in a significant increase in pressure-dependent tone in uterine arteries of pregnant animals. This hypoxia-mediated effect was inhibited by *N*-acetylcysteine (Fig. 4*B*).

N-Acetylcysteine restored hypoxia-induced down-regulation of the BK_{Ca} channel β1 subunit in uterine arteries

The β1 subunit plays a vital role in regulating BK_{Ca} channel activity in vascular smooth muscle by increasing the Ca^{2+} sensitivity of the channel (Hu & Zhang, 2012). Previous study revealed that long-term high- altitude hypoxia was associated with a selective down-regulation of the BK_{Ca} channel β1 subunit in uterine arteries of pregnant sheep (Hu *et al.* 2012). Consistent with this finding, the *ex vivo* treatment of uterine arteries of pregnant sheep with prolonged hypoxia showed a direct effect of hypoxia in suppressing expression of the BK_{Ca} channel β1 subunit protein in uterine arteries (Fig. 5). Of importance, the inhibition of ROS by *N*-acetylcysteine blocked the hypoxic effect and restored protein expression of the β1 subunit (Fig. 5). To confirm this finding of an *ex vivo* hypoxia-mediated effect in

animals exposed to chronic hypoxia, uterine arteries from pregnant sheep acclimatized to long-term high-altitude hypoxia were treated with *N*-acetylcysteine. As shown in Fig. 6*A*, *N*-acetylcysteine significantly increased protein abundance of the BK_{Ca} channel $\beta 1$ subunit in uterine arteries of pregnant sheep that had been exposed to long-term high-altitude hypoxia. Accordingly, the BK_{Ca} channel activity in uterine arterial myocytes of the hypoxic animals was significantly increased by *N*-acetylcysteine (Fig. 6*B*).

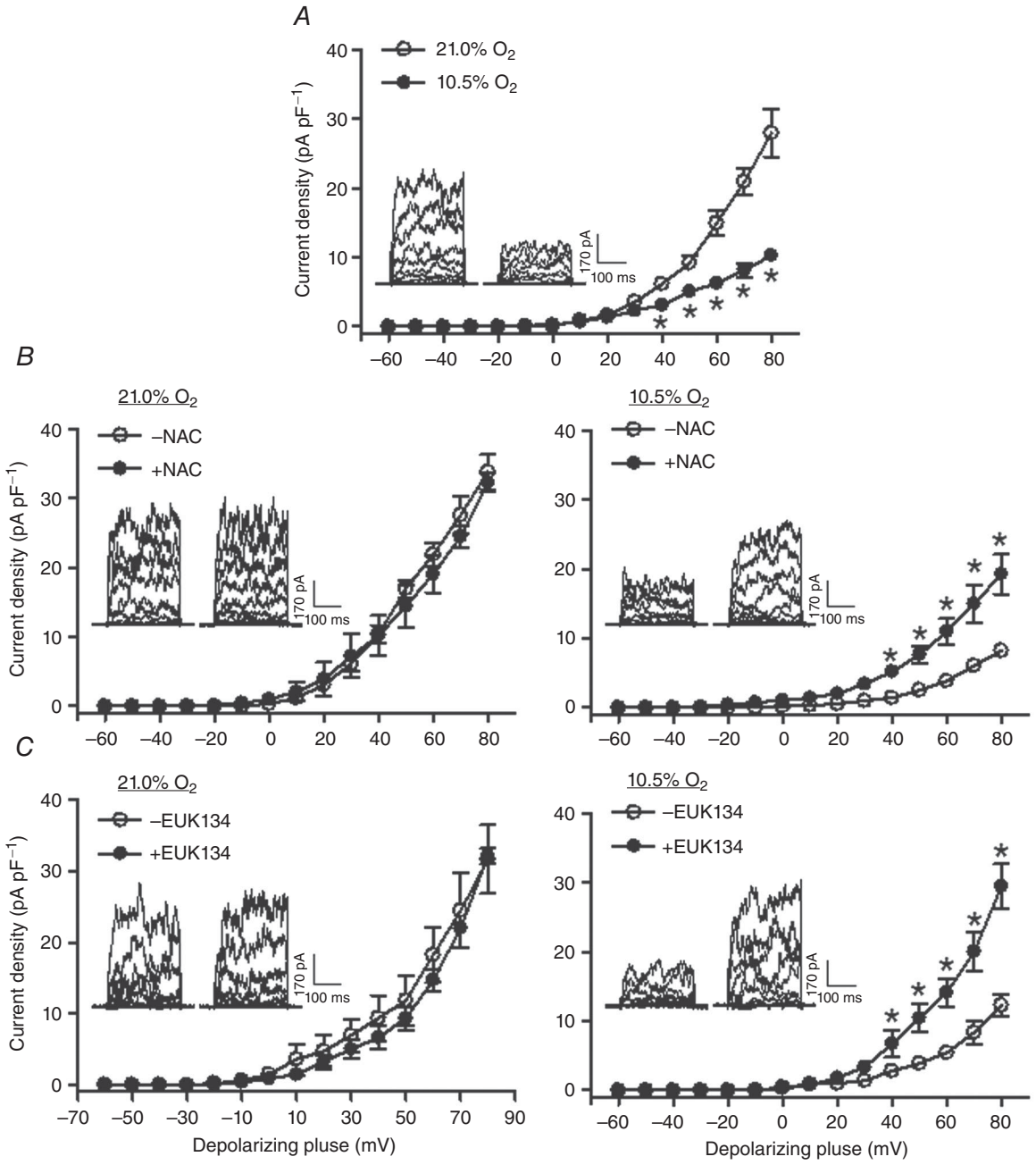

Figure 2. Antioxidants alleviated hypoxia-mediated inhibition of BK_{Ca} channel current density in uterine arteries

Uterine arteries of pregnant sheep were treated under control (21.0% O_2) and hypoxia (10.5% O_2) for 48 h in the absence or presence of 1 mM *N*-acetylcysteine (NAC) or 20 μM EUK 134. BK_{Ca} currents were normalized to cell capacitance and expressed as picoamps per picofarad (pA pF^{-1}; *y* axis), as a function of stepwise 10 mV depolarizing pulses (*x* axis). Data are means ± SEM of 5–7 cells from five animals of each group. Data were analysed with repeated measures ANOVA. $^*P < 0.05$, hypoxia *vs.* control in *A*; +NAC *vs.* –NAC in *B*; +EUK 134 *vs.* –EUK 134 in *C*.

Antioxidants reinstated steroid hormone-mediated up-regulation of BK_{Ca} channel activity under hypoxia

Steroid hormones, 17β-estradiol and progesterone, play a key role in up-regulating BK_{Ca} channel activity in uterine arteries during pregnancy (Hu *et al.* 2011). Under normoxic conditions, the steroid hormone treatment of uterine arteries from non-pregnant sheep for 48 h significantly increased BK_{Ca} channel current density (Fig. 7*A*). In contrast, under hypoxic conditions (10.5% O_2) in the absence of antioxidants, the hormonal treatment had no significant effect on BK_{Ca} channel activity (Fig. 7*B*). However, *N*-acetylcysteine and EUK 134 restored steroid hormone-mediated up-regulation of BK_{Ca} channel current density in uterine arteries under hypoxia (Fig. 7*C* and *D*). Accordingly, the previous study demonstrated that the hormonal treatment of uterine arteries from normoxic, non-pregnant sheep significantly increased NS1619-induced relaxations (Zhu *et al.* 2014). In contrast, the hormone treatment of uterine arteries from non-pregnant animals under hypoxic conditions had no significant effect on NS1619-induced relaxations (Fig. 8*A*). Of importance, *N*-acetylcysteine rescued the hormonal effect on the up-regulation of NS1619-induced relaxations under hypoxia (Fig. 8*B*).

Discussion

The present study presents several novel findings. (1) The *ex vivo* treatment of uterine arteries with prolonged hypoxia increased oxidative stress in the vessel, mimicking the effect observed *in vivo* in pregnant sheep acclimatized to long-term high-altitude hypoxia. (2) Hypoxia had a

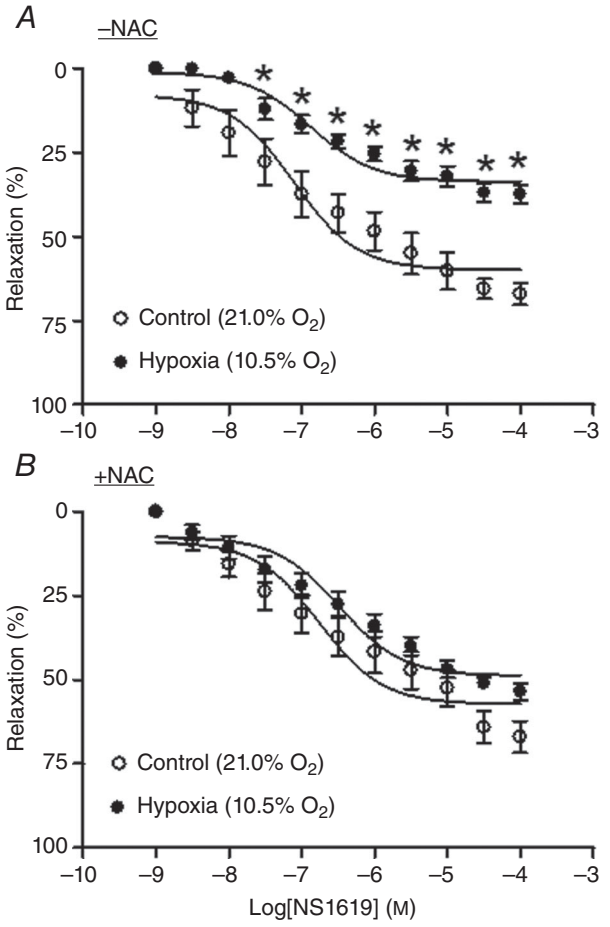

Figure 3. *N*-Acetylcysteine ablated hypoxia-mediated decrease in NS1619-induced relaxations in uterine arteries
Uterine arteries of pregnant sheep were treated under control (21.0% O_2) and hypoxia (10.5% O_2) for 48 h in the absence or presence of 1 mM *N*-acetylcysteine (NAC). NS1619-induced relaxations were determined after the treatments. Data are means ± SEM of five tissues from five animals of each group. Data were analysed with repeated measures ANOVA. $^*P < 0.05$, hypoxia *vs.* control.

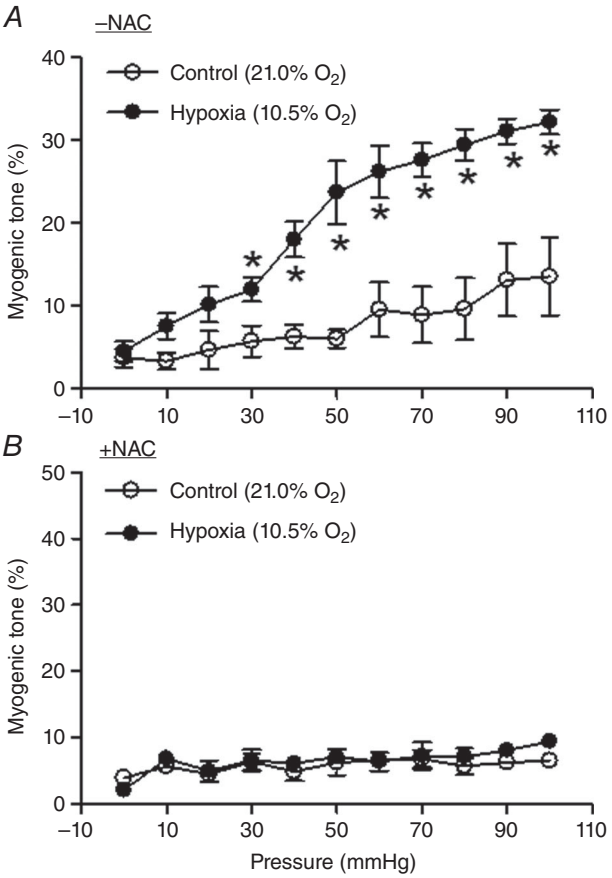

Figure 4. *N*-Acetylcysteine inhibited hypoxia-mediated increase in pressure-dependent tone in uterine arteries
Uterine arteries of pregnant sheep were treated under control (21.0% O_2) and hypoxia (10.5% O_2) for 48 h in the absence or presence of 1 mM *N*-acetylcysteine (NAC). Pressure-dependent tone was determined after the treatments. Data are mean ± SEM of four tissues from four animals of each group. Data were analysed with repeated measures ANOVA. $^*P < 0.05$, hypoxia *vs.* control.

direct effect on the down-regulation of BK_{Ca} channel β1 subunit expression and BK_{Ca} channel activity in uterine arteries, emulating the adverse effect of long-term high-altitude hypoxia on pregnant sheep. (3) Antioxidants ablated oxidative stress and blocked the hypoxia-induced inhibition of BK_{Ca} channels, providing a mechanistic understanding of oxidative stress as a causal role in the hypoxia-mediated effect. (4) Steroid hormone-induced up-regulation of BK_{Ca} channel function in uterine arteries was inhibited by hypoxia and this direct hypoxia-mediated effect was recovered by antioxidants, suggesting a target of hormonal effect by oxidative stress. (5) The impaired BK_{Ca} channel β1 subunit expression and channel activity in uterine arteries of pregnant sheep exposed to long-term high-altitude hypoxia were ameliorated by inhibiting oxidative stress, linking the findings of *ex vivo* and *in vivo* hypoxia-mediated effects and physiological significance in animals.

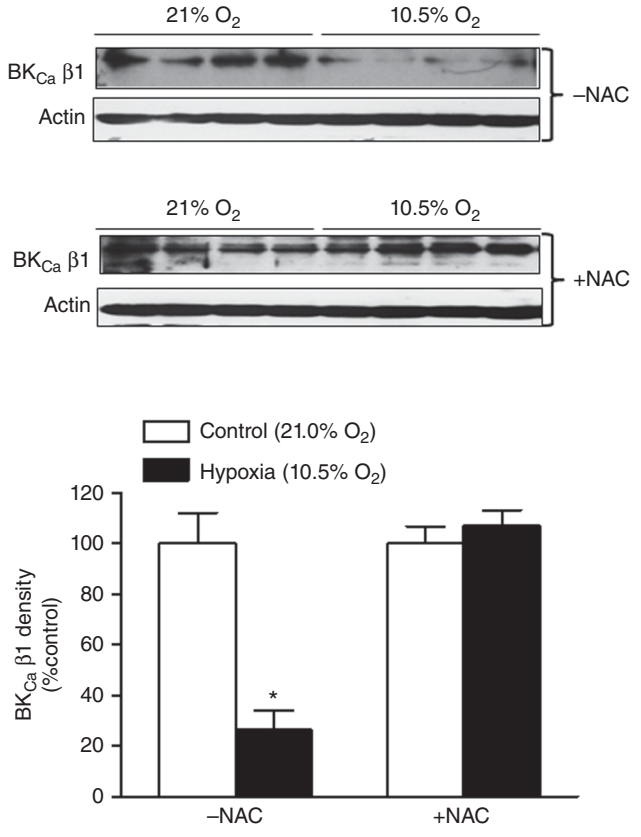

Figure 5. *N*-acetylcysteine restored hypoxia-induced down-regulation of the BK_{Ca} channel β1 subunit in uterine arteries
Uterine arteries of pregnant sheep were treated under control (21.0% O_2) and hypoxia (10.5% O_2) for 48 h in the absence or presence of 1 mM *N*-acetylcysteine (NAC). Protein abundance of the β1 subunit was determined by Western blot. Data are means ± SEM of four tissues from four animals of each group. Data were analysed by two-way ANOVA. $^*P < 0.05$, hypoxia *vs.* control.

Oxygen is a major determinant of gene expression in the cardiovascular system (Ratcliffe *et al.* 1998; Peers, 2002). Chronic hypoxia has been shown to cause down-regulation of BK_{Ca} channels in VSMCs (Bonnet *et al.* 2003; Navarro-Antolin *et al.* 2005). Consistent with these observations, we demonstrated that prolonged exposure of uterine arteries from pregnant sheep to hypoxia resulted in down-regulation of the BK_{Ca} channel β1 subunit. BK_{Ca} channels in VSMCs are primarily activated by an increase in intracellular Ca^{2+}

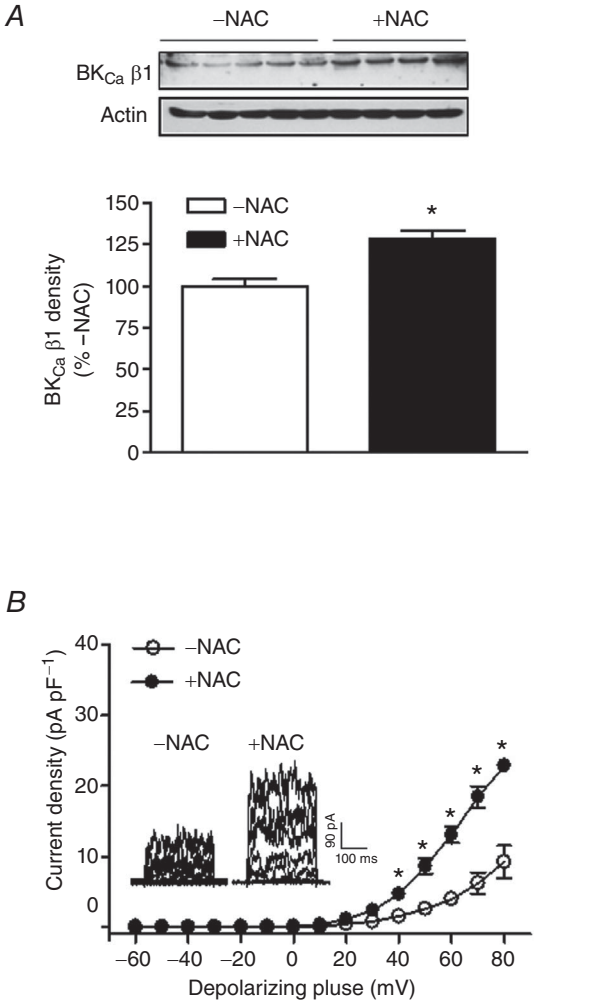

Figure 6. *N*-acetylcysteine increased the BK_{Ca} channel β1 subunit and BK_{Ca} channel in uterine arteries of high-altitude pregnant sheep
Uterine arteries isolated from pregnant sheep acclimatized to long-term high-altitude hypoxia were treated under 10.5% O_2 for 48 h in the absence or presence of 1 mM *N*-acetylcysteine (NAC). *A*, protein abundance of the β1 subunit was determined by Western blot. Data are means ± SEM of 4–5 tissues from 4–5 animals of each group. Data were analysed with *t* test. $^*P < 0.05$, +NAC *vs.* –NAC. *B*, BK_{Ca} currents in smooth muscle cells were recorded. Data are means ± SEM of six cells from five animals of each group. Data were analysed with repeated measures ANOVA. $^*P < 0.05$, +NAC *vs.* –NAC.

concentrations; and the association of the β1 subunit to the pore forming α subunit dramatically increases the channel's sensitivity to Ca^{2+} (Brenner *et al.* 2000; Hu & Zhang, 2012). The primary role of BK_{Ca} channels in the vasculature is to hyperpolarize the VSMC membrane and to promote vasorelaxation (Hu & Zhang, 2012). The impaired expression of the BK_{Ca} channel β1 subunit would in turn decrease the activation of BK_{Ca} channels, which could explain the reduced vasorelaxation mediated by BK_{Ca} channels and increased vascular pressure-dependent tone in uterine arteries of pregnant animals exposed to *ex vivo* hypoxia. However, the mechanism by which the BK_{Ca} channel β1 subunit is down-regulated following exposure to prolonged hypoxia is not completely understood. The similarity of adverse effects of long-term high-altitude hypoxia (Hu *et al.* 2012) and *ex vivo* hypoxia on BK_{Ca} channels suggests a direct effect of hypoxia in regulating BK_{Ca} channels in uterine arteries and that the *ex vivo* studies may provide an effective model to explore cellular and molecular mechanisms underlying aberrant uterine vascular function in response to high-altitude chronic hypoxia.

It is well established that ROS and oxidative stress play critical roles in the physiological and pathophysiological processes in the cardiovascular system (Taniyama & Griendling, 2003; Papaharalambus & Griendling, 2007). ROS include oxygen radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl ($^{\bullet}OH$) and non-radical hydrogen peroxide (H_2O_2); and superoxide and H_2O_2 are the major ROS in the cardiovascular system (Bedard & Krause, 2007; Papaharalambus & Griendling, 2007). ROS are generated through a cascade of chemical reactions that starts with the production of superoxide by the Nox family. Superoxide is short-lived and rapidly dismutated by SOD to H_2O_2, which is then converted by catalase to H_2O. Oxidative stress occurs as the result of an imbalance of production and removal of ROS. Chronic hypoxia promotes oxidative stress *in vitro* and *in vivo* and increases ROS in cultured VSMCs (Taniyama & Griendling, 2003; Papaharalambus & Griendling, 2007; Parraguez *et al.* 2011). In the present study, we demonstrated that *ex vivo* prolonged hypoxia treatment also promoted oxidative stress in uterine arteries. The level of oxidative stress in uterine arteries exposed to *ex vivo* hypoxia reported in the present study

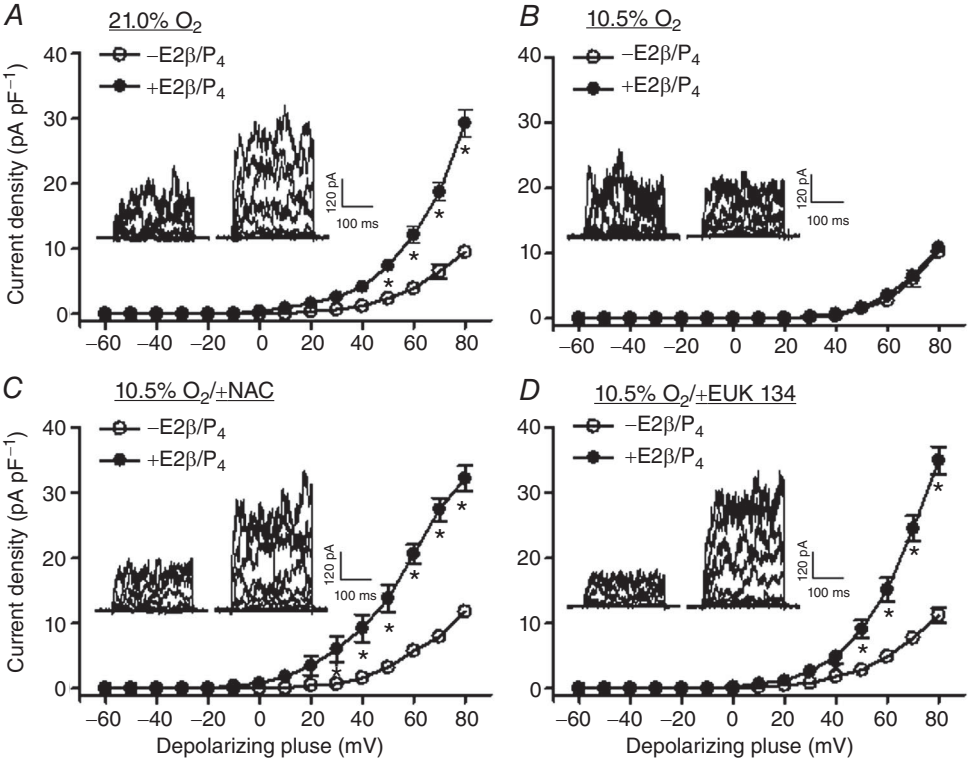

Figure 7. Antioxidants restored steroid hormone-mediated up-regulation of BK_{Ca} channel activity under hypoxia
Uterine arteries of non-pregnant sheep were treated with or without 17β-estradiol (E2β; 0.3 nM)/progesterone (P_4; 100 nM) under control (21.0% O_2) or hypoxia (10.5% O_2) for 48 h, in the absence or presence of 1 mM *N*-acetylcysteine (NAC) or 20 μM EUK 134. BK_{Ca} currents were normalized to cell capacitance and expressed as picoamps per picofarad (pA pF^{-1}; *y* axis), as a function of stepwise 10 mV depolarizing pulses (*x* axis). Data are means ± SEM of six cells from five animals of each group. Data were analysed with repeated measures ANOVA. $^*P < 0.05$; +E2β/P_4 *vs.* –E2β/P_4.

is comparable to the previous measurements in the vessels isolated from animals acclimatized to long-term high-altitude hypoxia (Xiao *et al.* 2013). Thus, our *ex vivo* culture condition largely mimicked high-altitude impacts on this vessel. High-altitude hypoxia elevated maternal oxidative stress in ewes (Parraguez *et al.* 2011), and gestational hypoxia promoted both maternal and placental oxidative stress in rats (Richter *et al.* 2012). It may be difficult to compare directly the data obtained from *ex vivo* studies and this *in vivo* study as different oxidative biomarkers were determined. Moreover, it is probable that locally produced ROS may play a predominant role in altering organ/tissue function. *N*-Acetylcysteine functions as an ROS scavenger through increasing intracellular glutathione and its thiol-disulfide exchange activity (Zafarullah *et al.* 2003). Although *N*-acetylcysteine may exert its action through non-antioxidant activities (Sun, 2010), the observations that the free radical scavenger *N*-acetylcysteine and SOD/catalase mimetic EUK 134 partially or completely reversed the effects of chronic hypoxia on the BK_{Ca} channel suggest that hypoxia-induced enhancement of ROS generation was primarily responsible for the aberrant BK_{Ca} channel function in uterine arteries. EUK 134 is a SOD/catalase mimetic and may not be able to discern oxidative species such as superoxide radicals and H_2O_2 that may be involved (Rong *et al.* 1999). Our recent findings that uterine arteries of high-altitude pregnant sheep exhibited increased oxidative stress in part by enhanced expression and activity of Nox2, and that an SOD mimetic tempol inhibited hypoxia-mediated increase in myogenic reactivity of uterine arteries (Xiao *et al.* 2013), would suggest a possible role of superoxide radicals in suppressing BK_{Ca} channel function in response to hypoxia. However, the possible involvement of H_2O_2 in impairing BK_{Ca} channel activity could not be excluded.

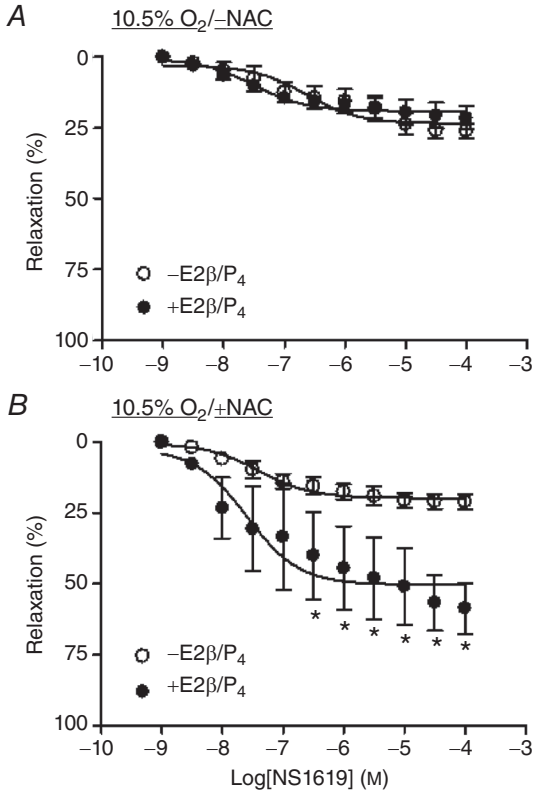

Figure 8. *N*-acetylcysteine recovered steroid hormone-mediated up-regulation of NS1619-induced relaxations in uterine arteries
Uterine arteries of non-pregnant sheep were treated with or without 17β-estradiol (E2β; 0.3 nM)/progesterone (P_4; 100 nM) under hypoxia (10.5% O_2) for 48 h, in the absence or presence of 1 mM *N*-acetylcysteine (NAC). NS1619-induced relaxations were determined after the treatments. Data are means ± SEM of five tissues from five animals of each group. Data were analysed with repeated measures ANOVA. $^*P < 0.05$; +E2β/P_4 *vs.* –E2β/P_4.

Increased ROS productions in cells could modify ion channel function through multiple mechanisms including post-translational modifications of key amino acid residues in channel proteins by oxidation and regulation of gene expression (Matalon *et al.* 2003). Our previous findings that acute application of *N*-acetylcysteine or the Nox inhibitor apocynin enhanced BK_{Ca} channel activity and the BK_{Ca} channel opener NS1619 induced relaxations in uterine arteries of high-altitude pregnant sheep (Xiao *et al.* 2013; Zhu *et al.* 2014) suggest that ROS could alter BK_{Ca} channel function by post-translational modification of the channel. The impairment of BK_{Ca} channel-mediated relaxation is unlikely to be due to endothelial damage as endothelium-dependent relaxation of this vessel was increased by chronic hypoxia, whereas endothelium-independent relaxation was not altered by chronic hypoxia (Xiao *et al.* 2001). BK_{Ca} channels in uterine arteries are inhibited by protein kinase C (PKC), and chronic hypoxia-induced upregulation of PKC has been shown to attenuate BK_{Ca} channel-mediated relaxation of uterine arteries in pregnancy (Hu *et al.* 2011; Xiao *et al.* 2014). Of interest, a chronic hypoxia-induced increase in PKC-mediated myogenic reactivity in uterine arteries of pregnant sheep was blocked by apocynin and tempol (Xiao *et al.* 2013).

In the present study, we extended our previous investigations and assessed the impact of oxidative stress induced by *ex vivo* hypoxia on BK_{Ca} channel expression and function in uterine arteries. We demonstrated that the direct effect of prolonged hypoxia induced an increase in oxidative stress and inhibition of BK_{Ca} channel β1 subunit expression in uterine arteries, and these effects were blocked by antioxidants. These findings suggest that oxidative stress caused by hypoxia plays a causal role in the down-regulation of the BK_{Ca} channel β1 subunit in uterine arteries. Furthermore, the findings that hypoxia-mediated suppression of BK_{Ca} channel activity decreases in BK_{Ca} channel-mediated vasorelaxation and increases in pressure-dependent tone all recovered by

antioxidants suggest that the dysfunction of BK_{Ca} channels in uterine arteries is probably secondary to BK_{Ca} channel repression conferred by increased oxidative stress. These observations support the notion that ROS play an important causative role in chronic hypoxia-induced BK_{Ca} channel repression and dysfunction in uterine arteries. Consistent with the present findings, oxidative stress was attributed to down-regulation of the BK_{Ca} channel β1 subunit in arteries, as well as SK_{Ca} and IK_{Ca} channels in atria of diabetic rodents (Lu *et al.* 2012; Zhao *et al.* 2014; Yi *et al.* 2015). Taken together, these studies provide evidence that the direct effect of chronic hypoxia regulates BK_{Ca} channel function in uterine arteries of pregnant animals via increased oxidative stress, involving both post-translational modification and gene expression. Downregulation of the BK_{Ca} channel β1 subunit by heightened oxidative stress may also result from alterations in protein turnover (i.e. translation and degradation). However, our recent finding that long-term high altitude drastically reduced Ca^{2+}-activated K^+ subunit beta-1 (KCNMB 1) mRNA level in uterine arteries from pregnant sheep (Chen *et al.* 2014) suggests that ROS generated during hypoxic exposure probably caused repression of the KCNMB 1 gene.

Both *in vivo* and *ex vivo* studies demonstrated that oestrogen alone or in conjunction with progesterone was able to imitate pregnancy-induced up-regulation of BK_{Ca} channel β1 subunit expression at both protein and mRNA levels and BK_{Ca} channel function in uterine arteries of non-pregnant sheep (Nagar *et al.* 2005; Hu *et al.* 2011; Zhu *et al.* 2014). However, the up-regulation of BK_{Ca} channel β1 subunit expression/function by steroid hormones was inhibited by chronic hypoxia (Hu *et al.* 2012; Chen *et al.* 2014; Zhu *et al.* 2014). In the present study, we showed that the oxidative stress promoted by chronic hypoxia in uterine arteries was responsible for dampening steroid hormone-stimulated up-regulation of BK_{Ca} channel function, as antioxidants were able to reinstate the hormonal upregulation of the BK_{Ca} channel. Similarly, we previously demonstrated that co-treatment of uterine arteries of high-altitude non-pregnant animals with steroid hormones and *N*-acetylcysteine restored BK_{Ca} channel activity and BK_{Ca} channel-mediated vasorelaxation (Zhu *et al.* 2014). *N*-Acetylcysteine probably restored the genomic actions of steroid hormones in upregulating BK_{Ca} channel expression and function in this study, although acute applications of oestrogen ($\geq$ 100 nM) could upregulate BK_{Ca} channel activity via non-genomic effects in VSMCs (White *et al.* 1995; Rosenfeld *et al.* 2000). The regulation of gene expression by oestrogen is primarily mediated by oestrogen receptors ER-α and ER-β in the cardiovascular system (Murphy, 2011). ER-α plays a critical role in oestrogen-mediated modulation of uterine vascular function, and both *in vivo* and *ex vivo* chronic hypoxia caused down-regulation of ER-α (Chang *et al.* 2010; Dasgupta *et al.* 2012). Oxidative stress has been demonstrated to down-regulate ER-α in endothelial cells (Chakrabarti & Davidge, 2009) and in a cancer cell line (Weitsman *et al.* 2009). This down-regulation could be triggered by post-translational modifications of ER-α serine residues (Weitsman *et al.* 2009) and/or epigenetic modification of the ER-α gene (Dasgupta *et al.* 2012). The diminished effect of steroid hormones in regulating BK_{Ca} channel function following exposure to chronic hypoxia is probably the consequence of the down-regulation of ER-α. In addition, oxidative stress has been shown to inhibit the binding of ERs to DNA (Liang *et al.* 1998), thus disrupting oestrogen-stimulated transcription of the KCNMB 1 gene. Nevertheless, reducing oxidative stress may prevent the adverse effects of chronic hypoxia on ER-α and reinstate the ability of steroid hormones to upregulate BK_{Ca} channel expression and function in uterine arteries.

The finding that the antioxidants were able to suppress chronic hypoxia-induced down-regulation of BK_{Ca} channel expression and function in uterine arteries is appealing. High-altitude hypoxia has been shown to reduce uterine blood flow and is associated with increased incidence of pre-eclampsia and fetal growth restriction (Zamudio *et al.* 1995*a*,*b*; Palmer *et al.* 1999; Browne *et al.* 2011). Compelling evidence indicates that oxidative stress plays an important role in the pathogenesis of pregnancy complications including pre-eclampsia and fetal growth restriction (Roggensack *et al.* 1999; Raijmakers *et al.* 2004; Siddiqui *et al.* 2010; Stanley *et al.* 2012). Recent studies revealed that Nox 2 expression was upregulated in umbilical vessels and primary cultured human umbilical vein endothelial cells from pre-eclamptic pregnancy (Choi *et al.* 2013; Lim *et al.* 2015). It appeared that Nox 2-derived ROS caused down-regulation of K_{Ca}3.1 in these vessels (Choi *et al.* 2013). Interestingly, we also demonstrated upregulation of Nox 2 in uterine arteries from sheep acclimatized to long-term high altitude (Xiao *et al.* 2013) and development of pre-eclampsia-like symptoms in pregnant rats following gestational hypoxia (Zhou *et al.* 2013). Our findings raise the possibility that ROS-induced impairment of BK_{Ca} channel function in uterine arteries could be attributed to the pathogenesis of these complications. Whereas it may be relatively difficult to directly test this link *in vivo* by inhibiting Nox 2-derived ROS in sheep exposed to long-term high-altitude hypoxia, rodents may provide an alternative model to further investigate this mechanism *in vivo* in future studies. Our recent study demonstrated that gestational hypoxia induced pre-eclampsia-like symptoms in pregnant rats (Zhou *et al.* 2013). Interestingly, chronic treatment with the ROS scavenger tempol in mouse models of fetal growth restriction and pre-eclampsia improved fetal growth, which was associated with an increase in uterine artery blood flow (Hoffmann *et al.*

2008; Stanley *et al.* 2012). Moreover, supplementation with antioxidant oxidants such as vitamins C and E during pregnancy effectively prevents maternal oxidative stresses triggered by high-altitude and gestational hypoxia, improving pregnancy outcomes (Parraguez *et al.* 2011; Richter *et al.* 2012). Similarly, the antioxidants *N*-acetylcysteine and tempol have been shown to alleviate hypertension produced by reduced uterine perfusion in pregnant rats, an animal model mimicking pre-eclampsia (Chang *et al.* 2005; Sedeek *et al.* 2008). Additionally, ROS-scavenging therapies improved voltage-gated K^+ (K_V) channel function in pulmonary arteries of newborn pigs with progressive hypoxia-induced pulmonary hypertension (Fike *et al.* 2013) and reduced hypoxia-induced pulmonary hypertension and right ventricular hypertrophy in rats (Hoshikawa *et al.* 2001). Thus, antioxidant treatment may provide a useful intervention to improve impaired uteroplacental function associated with pregnancy complications. However, note that minimal levels of ROS are necessary for proper cell function in early fetal development, and wide use of antioxidants should be avoided at this stage.

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Additional information

Conflict of interest

None.

Author contributions

X.Q.H.: conceived, designed and performed experiments, analysed data, and wrote the manuscript. X.H.: performed experiments and analysed data. D.X.: performed experiments, analysed data and participated in writing the manuscript. L.Z.: provided animals and all other support for the studies, conceived and designed the experiments, interpreted data and wrote the manuscript. All contributors have read and approved the final manuscript for submission.

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