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RESEARCH PAPER

Milrinone attenuates thromboxane receptor-mediated hyperresponsiveness in hypoxic pulmonary arterial myocytes

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BACKGROUND AND PURPOSE

Neonatal pulmonary hypertension (PPHN) is characterized by pulmonary vasoconstriction, due in part to dysregulation of the thromboxane prostanoid (TP) receptor. Hypoxia induces TP receptor–mediated hyperresponsiveness, whereas serine phosphorylation mediates desensitization of TP receptors. We hypothesized that prostacyclin (IP) receptor activity induces TP receptor phosphorylation and decreases ligand affinity; that TP receptor sensitization in hypoxic myocytes is due to IP receptor inactivation; and that this would be reversible by the cAMP-specific phosphodiesterase inhibitor milrinone.

EXPERIMENTAL APPROACH

We examined functional regulation of TP receptors by serine phosphorylation and effects of IP receptor stimulation and protein kinase A (PKA) activity on TP receptor sensitivity in myocytes from neonatal porcine resistance pulmonary arteries after 72 h hypoxia *in vitro*. Ca^{2+} response curves to U46619 (TP receptor agonist) were determined in hypoxic and normoxic myocytes incubated with or without iloprost (IP receptor agonist), forskolin (adenylyl cyclase activator), H8 (PKA inhibitor) or milrinone. TP and IP receptor saturation binding kinetics were measured in presence of iloprost or 8-bromo-cAMP.

KEY RESULTS

Ligand affinity for TP receptors was normalized *in vitro* by IP receptor signalling intermediates. However, IP receptor affinity was compromised in hypoxic myocytes, decreasing cAMP production. Milrinone normalized TP receptor sensitivity in hypoxic myocytes by restoring PKA-mediated regulatory TP receptor phosphorylation.

CONCLUSIONS AND IMPLICATIONS

TP receptor sensitivity and EC_{50} for TP receptor agonists was regulated by PKA, as TP receptor serine phosphorylation by PKA down-regulated Ca^{2+} mobilization. Hypoxia decreased IP receptor activity and cAMP generation, inducing TP receptor hyperresponsiveness, which was reversed by milrinone.

Abbreviations

EP, prostaglandin E receptor; IP, prostacyclin receptor; IP_3, inositol-1,4,5-trisphosphate; NECA, adenosine-5′-N-ethylcarboxamide; PDE, phosphodiesterase; PKA, protein kinase A; TP, thromboxane receptor

Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a multifactorial disorder of postnatal pulmonary vasoconstriction and remodelling, resulting in respiratory failure, hypoxemia and 20% mortality. Common PPHN aetiologies include meconium aspiration (nearly 40% of cases) and sepsis (25%) (Clark *et al.*, 2000; Konduri *et al.*, 2004; Finer and

Barrington, 2006). Infants with PPHN who have sepsis or inflammation are among the poorest responders to vasodilators.

The crucial endogenous pathways that regulate perinatal pulmonary vascular tone include the nitric oxide–endothelin and prostacyclin–thromboxane systems (Weinberger *et al.*, 2001). Thromboxane A_2 (TxA_2), an inflammatory prostanoid with vasoconstrictive and mitogenic properties, contracts pulmonary arterial myocytes via the $G\alpha_q$-coupled thromboxane receptor (TP) (nomenclature follows Alexander *et al.*, 2009), leading to increased intracellular [Ca^{2+}], force generation and sensitization of the contractile apparatus to Ca^{2+} (Cogolludo *et al.*, 2003). TxA_2 is abundant in the neonatal circulation at birth, participating in the closure of the umbilical vessels and the ductus arteriosus (Reyes, 1993). Hypoxic PPHN increases the serum TxA_2 to prostacylin (PGI_2) ratio (Fike *et al.*, 2003). Hypoxia increases pulmonary vasoconstriction to the TP receptor agonist U46619 by over fivefold, relative to its effect under normoxic conditions, and underlies hypersensitization to agonist ligands (Snow *et al.*, 2008). Hypoxia has a priming effect on pulmonary arterial myocytes, increasing inositol 1,4,5-trisphosphate (IP_3) generation in response to vasoconstrictors (Peacock *et al.*, 1998).

Postnatal prostacyclin (PGI_2) production is normally high, but hypoxia inverts the TxA_2 : PGI_2 ratio by decreasing prostacyclin synthesis (Fike *et al.*, 2003). PGI_2 mediates vasodilatory and anti-inflammatory actions via the prostacyclin (IP) receptor, which is coupled via $G\alpha_s$ to adenylyl cyclase (Vane and Botting, 1995). Clinical use of prostacyclin in pulmonary hypertension is limited by rapid agonist-induced down-regulation of the IP vasodilator response (Sobolewski *et al.*, 2004).

Phosphorylation is a primary regulatory mechanism for vascular prostanoid receptors, in response to ligand binding, or due to signalling interactions with other prostanoid receptors (Breyer *et al.*, 2001). TP receptor phosphorylation is induced by pulmonary vasodilators, including PGI_2 (Reid and Kinsella, 2003). IP receptor–mediated phosphorylation of TP receptors has been ascribed to protein kinase C (PKC) (Kelley-Hickie and Kinsella, 2004) and protein kinase A (PKA) (Walsh *et al.*, 2000; Reid and Kinsella, 2003) and leads to TP receptor desensitization (Reid and Kinsella, 2003; O'Meara and Kinsella, 2004) and internalization (Parent *et al.*, 1999). The ligand affinity of human TP receptor isoforms is regulated by PKA-mediated phosphorylation at serine 329 (Habib *et al.*, 1997; Foley *et al.*, 2001; Reid and Kinsella, 2003).

We reported that in normal neonatal pulmonary artery myocytes, TP receptors are maintained in a state of low affinity by tonic phosphorylation. Hypoxia decreases inhibitory TP receptor phosphorylation, causing hypersensitivity of these receptors. Calcium mobilization in pulmonary arterial myocytes is increased after hypoxia both *in vivo* and *in vitro*, with sustained effects even after subsequent cell culture in normoxia (Hinton *et al.*, 2006). Hypoxic sensitization of TP receptors occurs due to a decreased ligand K_d shown as a shift of the agonist competitive binding curve to the left (Hinton *et al.*, 2007), indicating an increased TP receptor affinity for agonists (Gong *et al.*, 2010).

Milrinone, a phosphodiesterase (PDE)-3 inhibitor, is an inotrope and vasodilator, increasing cellular cAMP and improving hypoxic pulmonary haemodynamics (Joynt *et al.*, 2008; Lakshminrusimha *et al.*, 2009). Studies on the functional effects of milrinone have recently focused on prostanoid receptors, as milrinone attenuates inflammatory lung injury (Bueltmann *et al.*, 2009), potentiates the anti-remodelling effect of PGI_2 on hypoxic artery (Phillips *et al.*, 2005) and enhances relaxation to PGI_2 in PPHN (Lakshminrusimha *et al.*, 2009).

In this study, our objective was to study whether the loss of TP receptor regulation after hypoxia *in vitro* may be due to diminished activity of PKA. We hypothesized that hypoxia inhibits IP receptor signalling and depresses myocyte PKA activity, thereby inhibiting TP receptor phosphorylation and enhancing TP receptor affinity. Since milrinone increases PKA activity, we further hypothesized that sensitization of TP receptors induced by hypoxia would be reversed by milrinone treatment, reactivating PKA-mediated TP receptor regulation.

Methods

All animal care and experimental protocols were approved by the University of Manitoba Central Animal Care committee, in accordance with Canadian Council on Animal Care and US National Institutes of Health guidelines. Cultured myocytes from resistance pulmonary arteries of newborn (<24 h age) swine were examined in first passage, following 72 h normoxic or hypoxic exposure.

Cell culture

Pulmonary artery smooth muscle cells were obtained from newborn swine using a dispersed cell culture method selective for myocytes (Shimoda *et al.*, 2000). Fourth- to sixth-generation pulmonary arteries were obtained by microdissection into Ca^{2+}-free Krebs–Henseleit physiological buffer and were allowed to recover in cold HEPES-buffered saline solution (HBS; in mM: 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4) supplemented with antibiotic/antimycotic mixture and gentamicin. Arteries were washed in Ca^{2+}-reduced HBS (20 μM $CaCl_2$), finely minced, then the tissue transferred to a digestion solution containing Ca^{2+}-reduced HBS, type I collagenase (1750 U·mL^{-1}), dithiothreitol (1 mM), BSA (2 mg·mL^{-1}) and papain (9.5 U·mL^{-1}) for 15 min at 37°C with gentle agitation. Dispersed myocytes were collected by centrifugation at 800× *g* for 5 min, washed in Ca^{2+}-free HBS to remove digestion solution and then resuspended in culture medium. Cells were plated at a density of 4.4×10^4 cells cm^{-2} in Ham's F-12 medium with L-glutamine supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin. Once confluent, myocytes were serum-deprived for 2 days (Ham's F-12 with L-glutamine/penicillin/streptomycin and 1% insulin–transferrin–selenium) to synchronize cells in a contractile phenotype, then split into two groups for the final 3 days of culture: (i) control normoxic myocytes, maintained serum-free in 21% O_2, 5% CO_2; and (ii) hypoxic myocytes, maintained serum-free in 10% O_2, 5% CO_2 for 3 days.

Live cell calcium mobilization

Myocytes were rinsed free of media in Hanks balanced salt solution (HBSS; in mM: 1.26 $CaCl_2$, 0.493 $MgCl_2$·6 H_2O, 0.407

$MgSO_4 \cdot 7\ H_2O$, 5.33 KCl, 0.441 KH_2PO_4, 4.17 $NaHCO_3$, 137.93 NaCl, and 0.338 $NaHPO_2$) with 0.1% BSA. Myocytes were loaded with the Ca^{2+}-sensitive fluorescent dye fura 2-acetoxymethyl ester (fura-2AM) dissolved in dimethyl sulphoxide, as 5 μM in an HBSS/0.1% BSA solution, with 1.0 $\mu g \cdot mL^{-1}$ pluronic acid (for AM ester solubilization), for 1 h at 37°C. Extracellular fura-2AM was washed off with HBSS/0.1% BSA. Cells were allowed to recover for 30 min at room temperature, for complete cleavage of intracellular AM esters. Coverglass plates were secured on an inverted microscope (Olympus, Markham, Ontario, Canada) in 21% O_2, and studied at 20× magnification. Cells were challenged with serial concentrations of the TP receptor agonist U46619 after pre-incubation with or without 10 μM forskolin (adenylyl cyclase activator), 1 μM H8 (PKA inhibitor), 1 μM iloprost (IP receptor agonist) for 1 h; or 1 μM deoxy-prostaglandin-E_1 (stable, partially selective EP_2 receptor agonist), 0.1 μM adenosine-5′-N-ethylcarboxamide (NECA, selective adenosine A_{2B} receptor agonist) or 5 nM milrinone for 15 min. Ratiometric imaging of intracellular Ca^{2+} concentration utilized excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm; data were captured by a charge-coupled device camera and Perkin Elmer software (Montreal, Quebec, Canada). Each recording consisted of a stable baseline and a return to same. Following subtraction of baseline fluorescence, Ca^{2+} mobilization was analyzed from at least four equally sized regions from each microscope field, tracing three to five cells with minimal cell-free areas. Emission ratios from 340/380 λ excitations were quantified against a calcium standard (Grynkiewicz *et al.*, 1985).

PKA assay

PKA activity was measured in a 96-well ProFluor assay kit (Promega, Nepean, Ontario, Canada), using PKA substrate peptide bisamide rhodamine 110 (PKA R110, 25 μL) pre-incubated with 25 μL ATP reagent in reaction buffer. Following 10 min incubation with 1 μM 8-bromo-cAMP, 1 μM H8 (Maggi *et al.*, 1996), 1 μM iloprost, 10 nM milrinone or diluent, lysates from hypoxic and normoxic pulmonary artery myocytes were added to the reaction mixture and incubated for 3 min. Reaction was terminated with 25 μL protease reagent in termination buffer. After a 30 min incubation, and addition of 25 μL stabilizing reagent, fluorescence was measured at 485 nm excitation and 530 nm emission λ, against reagent buffer blanks, using a FLUOstar Optima microplate reader (BMG Labtec GMBH, Hanns-Martin-schleyer-strio, Offenburg, Germany). Phosphorylated PKA R110 substrate is resistant to digestion by the protease reagent and remains non-fluorescent; fluorescence thus inversely correlates with PKA activity. Measured fluorescence was inverted to calculate PKA activity (arbitrary units) and expressed as fold-change compared to (untreated) normoxic lysates.

Sample preparation for receptor kinetics

Myocytes were pre-treated for 24 h with 1 μM U46619, 1 μM iloprost, 1 μM 8-bromo cAMP or diluent. Cells were twice rinsed in PBS, then lysed in binding buffer (25 mM Tris, 10 mM $CaCl_2$, 0.01 mM indomethacin and 75 $\mu g \cdot mL^{-1}$ PMSF, pH 7.4). Unlysed cells and particulate matter were removed by brief centrifugation at 2000× *g*. The supernatant was then centrifuged at 100 000× *g* for 60 min at 4°C, and membrane fractions were resuspended in binding buffer. Aliquots (30–60 μg protein) of this membrane preparation were used for radioligand experiments.

Saturation binding kinetics

Saturation binding kinetics was quantified in membrane fractions of hypoxic and normoxic myocytes pre-treated for 24 h with 1 μM U46619, 1 μM iloprost or 1 μM 8-bromo-cAMP. Samples were incubated with ^{3}H-SQ-29548 (0.1–50 nM; diluted in binding buffer) with or without an excess of unlabelled U46619 (10 μM), in a total reaction volume of 100 μL for 1 h at room temperature. Reactions were terminated by vacuum filtration, and membranes were washed twice with ice-cold binding buffer. Filters were agitated in 500 μL distilled water to release adsorbed radioisotope and then allowed to equilibrate in 5 mL CytoScint (ICN) for 5 h before counting. Unbound radioisotope was also collected. Counts per minute were analyzed for 3 min per sample.

PGI_2 receptor (IP) abundance

Hypoxic and normoxic myocytes were scraped and sonicated in 300 μL ice-cold radioimmunoprecipitation assay buffer (PBS containing 0.1%SDS, 1% Triton X100, 1 mM EDTA, 3.3 mM PMSF, 10 mM sodium orthovanadate and protease inhibitor [4-(2-aminoethyl) benzenesulfonyl fluoride] 0.1 mM, aprotinin 0.08 μM, bestatin 4 μM, N-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide 1.4 μM, leupeptin 2.2 μM, pepstatin A 1.5 μM). IP receptor protein abundance was measured in 20 μg protein by Western blot following SDS-PAGE on polyvinylidene fluoride membrane, using polyclonal rabbit antibody to human IP receptors (Cayman Chemical, Ann Arbor, MI, USA) at 1:250 dilution overnight and visualized after incubation with 1:5000 goat anti-rabbit IgG peroxidase conjugate.

cAMP assay

Cells were treated with 1 μM iloprost for serial time intervals. Media was removed, and cytosolic protein was precipitated with acidic ethanol. Samples were separated by brief centrifugation at 5000× *g*, then supernatant was neutralized with KOH prior to cAMP assay. cAMP was measured using an assay kit (TRK 432, Amersham/GE, Baie d'urfe, Quebec, Canada), based on competitive binding between unlabelled cAMP and a fixed quantity of the supplied ^{3}H-labelled binding protein with high specificity and affinity for cAMP. Results were normalized to extract protein content.

PDE assay

cAMP-specific PDE assay used reagents provided in QuantiZyme Cyclic Nucleotide PDE colorimetric assay kit (Biomol, Burlington, Ontario, Canada), with a modified protocol to assess cAMP degradation due to lysate enzyme activity. Normoxic and hypoxic myocytes were incubated for 5 min with selective PDE inhibitors: 1 μM vinpocetine (PDE-1-selective; Enzo Life Sciences, Burlington, Ontario, Canada), Bay-60-755 (PDE-2; Cayman Chemical), milrinone (PDE-3), rolipram (PDE-4; Sigma, Oakville, Ontario, Canada) or diluent. Plates were then washed twice with PBS, and cells were scraped into

lysis buffer containing protease and phosphatase inhibitors. Cytosolic fractions were isolated by fractional centrifugation. The assay mixture, containing 20 µL of 0.5 mM cAMP substrate, 75 µg cytosolic protein and assay buffer, was incubated at 30°C for 30 min. Reactions were terminated by the addition of Biomol Green reagent. AMP resulting from the action of cytosolic PDE was degraded by 5′-nucleotidase to release free phosphate, generating a colorimetric reaction quantified at 620 nm in a microplate reader (against a blank containing cytosolic protein without substrate). Phosphate release per unit time was calculated from a linear standard curve generated using 5′-AMP concentrations of 0.05–3.0 nM and 5′-nucleotidase, normalized to lysate protein content (Kolosionek *et al.*, 2009).

IP_3 measurement

Confluent serum-starved normoxic and hypoxic pulmonary arterial myocytes were pre-treated for 30 min with 1 µM iloprost, 10 nM 8Br-cAMP or diluent, then challenged with 1 µM U46619 for 1 min. Unstimulated controls were used to determine basal levels of IP_3. Cells were lysed, and intracellular IP_3 was extracted by ice-cold 20% trichloracetic acid for 20 min. Precipitated proteins were sedimented by centrifugation; supernatants were collected and neutralized with 10 M KOH. IP_3 was quantified using a TRK1000 radio-competition binding assay employing [^{3}H]-D-myo-IP_3 as the radioactive standard (GE Life Sciences, Baie d'urfe, Quebec, Canada) and expressed as pmol IP_3 mg^{-1} lysate protein (measured in the pelleted cellular extract).

Statistical analyses

Quantitative data were analysed by ANOVA. Ca^{2+} mobilization was calculated from measured 340/380 nm emission ratios, and log EC_{50} determined by sigmoidal curve fit of transformed data. Data are expressed as mean ± SD or mean ± SEM; $P < 0.05$ was taken to be significant.

Materials

^{3}H SQ29548 (0.1 mCi·mL^{-1}) was obtained from Perkin Elmer and ^{3}H iloprost (0.1 mCI·mL^{-1}) from GE Life Sciences. Forskolin, 8-Br-cAMP, U46619, milrinone, NECA, rolipram and indomethacin were from Sigma Aldrich (Oakville, Ontario, Canada); fura-2AM from Molecular Probes (Burlington, Ontario, Canada); iloprost, Bay-60 and 11-deoxy-PGE_1 from Cayman Chemicals; H8 and vinpocetine from Biomol Enzo Life Sciences.

Results

Hypoxia causes thromboxane receptor hypersensitivity and hyperreactivity

Neonatal porcine pulmonary arterial myocytes in the first passage were loaded with the calciometric dye fura-2AM and challenged with increasing concentrations of TP receptor agonist U46619, following 72 h exposure to hypoxia, or normoxia *in vitro*. The dose–response relation for U46619 in hypoxic myocytes was shifted to the left (hypersensitive) and upwards (hyperreactive), compared with that for normoxic myocytes (Figure 1A), resulting in sensitization of the normalized dose–response curve and a lower concentration required for 50% maximal response (Figure 1B; EC_{50} in hypoxic myocytes 0.53 µM and in normoxic myocytes 1.2 µM; $P < 0.0005$). Both hypersensitivity and hyperreactivity are elements of pharmacological hyperresponsiveness.

Effect of hypoxia on PKA activity

Hypoxia decreases PKA activity in myocyte lysates by nearly 40% (Figure 2). Treatment with the cAMP analogue, 8-bromo-cAMP (1 µM) or with the PDE-3 inhibitor milrinone (1 µM), increased PKA activity in hypoxic myocytes to normoxic levels, while treatment with the non-selective PKA inhibitor H8 (1 µM) decreased normoxic PKA activity, to the level observed in untreated hypoxic myocytes.

Effect of PKA activation state on TP receptor sensitivity

We examined regulation of TP receptorsensitivity by adenylyl cyclase, or its downstream effector PKA. Calcium mobiliza-

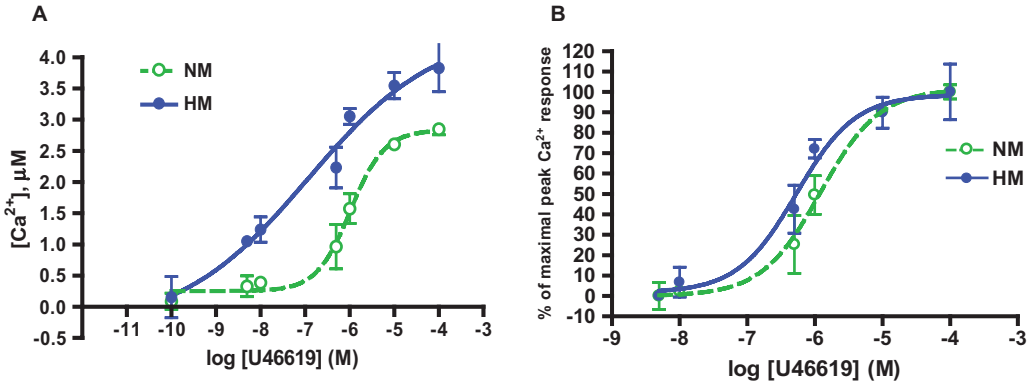

Figure 1

Hypoxia causes TP receptor hypersensitivity and hyperreactivity. (A) Hypoxic (HM) and normoxic (NM) pulmonary arterial myocytes were loaded with fura-2AM and challenged with TP receptor agonist U46619. Peak calcium mobilization calculated from 340/380 nm emission ratio. HM calcium response is left-shifted (hypersensitive) and up-shifted (hyperreactive) relative to NM curve. (B) Concentration response curve for U46619, standardized to maximal response. NM EC_{50}, 1.2 µM; HM EC_{50}, 0.53 µM (data in curves presented as mean ± SD; $P < 0.0005$, $n = 15$).

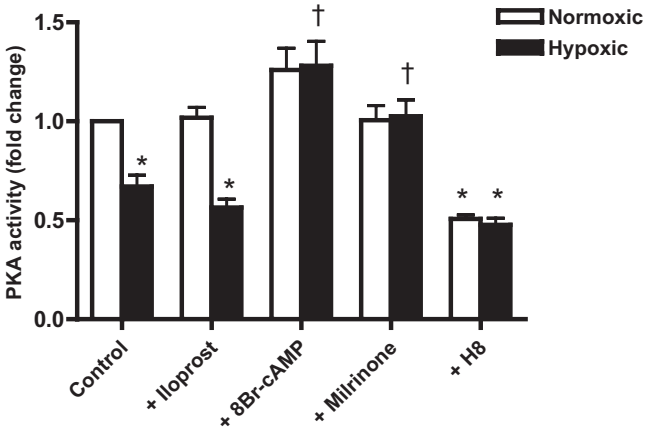

Figure 2

Hypoxia decreases PKA activity. PKA activity determined in myocyte lysates obtained following pre-incubations with 1 μM iloprost, 8-bromo-cAMP or H8, 10nM milrinone or diluent. Mean ± SEM; n = 6–8 separate experiments; *P < 0.05 compared with normoxic untreated myocytes; †P < 0.05 compared with hypoxic untreated myocytes.

tion was quantified to serial concentrations of U46619 in hypoxic and normoxic myocytes pre-incubated for 15 min with forskolin, H8 or buffer. Forskolin had no effect on responses of normoxic myocytes (Figures 3A and 4A) but shifted the response of hypoxic myocytes to U46619 to the right, resulting in normalization of the dose–response curve for hypoxic myocytes (Figures 3C and 4C). H8 induced a leftward displacement of the U46619 response curve in normoxic myocytes, towards the position of the curve for hypoxic myocytes (Figures 3B and 4B) while causing no further change in response of hypoxic myocytes (Figures 3D and 4D). EC_{50}s for each condition were calculated from percent (normalized) dose–response curves. All dose–response relationships in conditions of phosphorylated TP receptors showed an approximately threefold increase in EC_{50} (Table 1).

TP receptor kinetics

TP receptor saturation binding kinetics were determined in cell membrane fractions from hypoxic and normoxic myocytes, using ^{3}H-SQ29548, a TP receptor–specific antagonist (Figure 5A). The dissociation constant (slope or K_d, in nM) and the maximal concentration of available binding sites (B_{max}, expressed as fmoL·mg^{-1}) were significantly decreased in hypoxic, compared with normoxic myocytes (Table 2). Incubation of cell extracts with the IP receptor agonist iloprost (1 μM) markedly desensitized TP receptors, increasing K_d in hypoxic and normoxic myocytes (Figure 5B, P < 0.01), and eliminating the difference between these groups (Table 2).

IP receptor abundance, activity and cAMP generation

The effect of the IP agonist iloprost (1 μM) on intracellular cAMP was examined in a time-dependent study, under

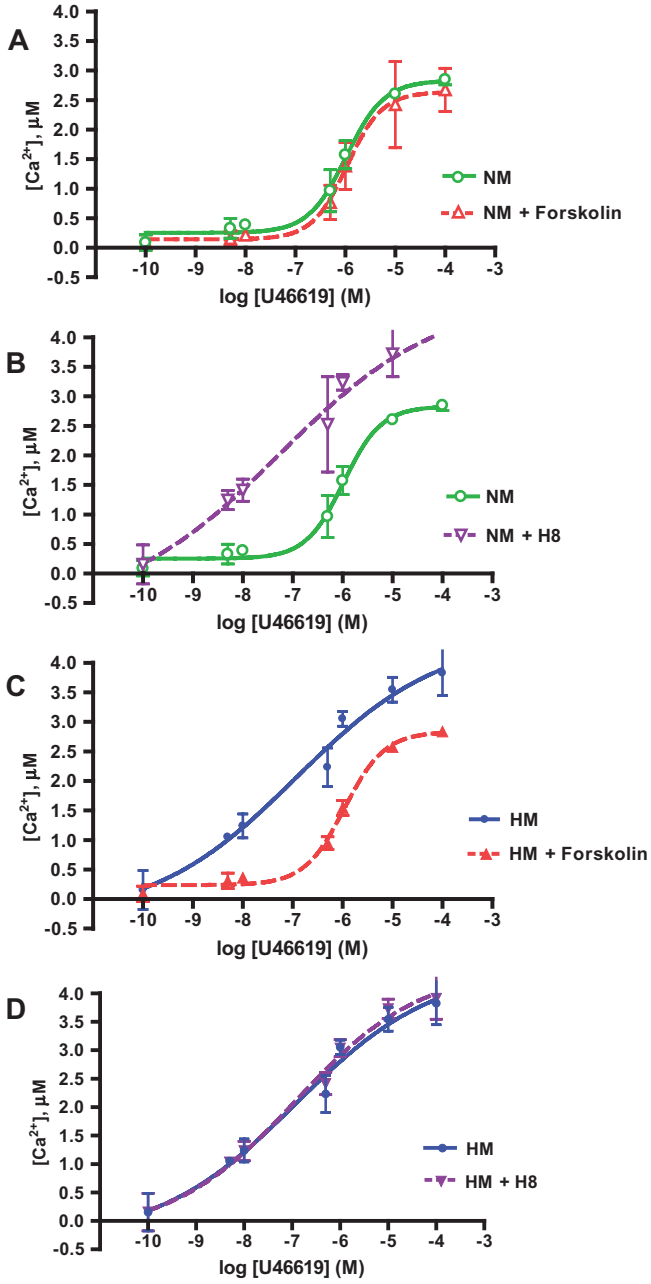

Figure 3

PKA activation state determines TP receptor reactivity and maximal calcium mobilization. Concentration response curves for TP receptor agonist U46619, showing peak Ca^{2+} response to U46619 challenge quantified in fura-2AM-loaded pulmonary arterial myocytes pre-incubated for 15 min with 1 μM forskolin (activating PKA) or H8 (inactivating PKA), or buffer. Baseline Ca^{2+} subtracted from all peak measurements. All curves presented as mean ± SD; n = 15–20 per point. (A) Forskolin has no effect on the dose–response curve to U46619 in normoxic myocytes (NM). (B) H8 shifted the curve in NM to the left and upwards to resemble the curve in hypoxic myocytes (HM). (C) Forskolin exposure normalizes TP receptor hyperresponsiveness in HM. (D) H8 has no effect on the dose–response to the TP receptor agonist in HM.

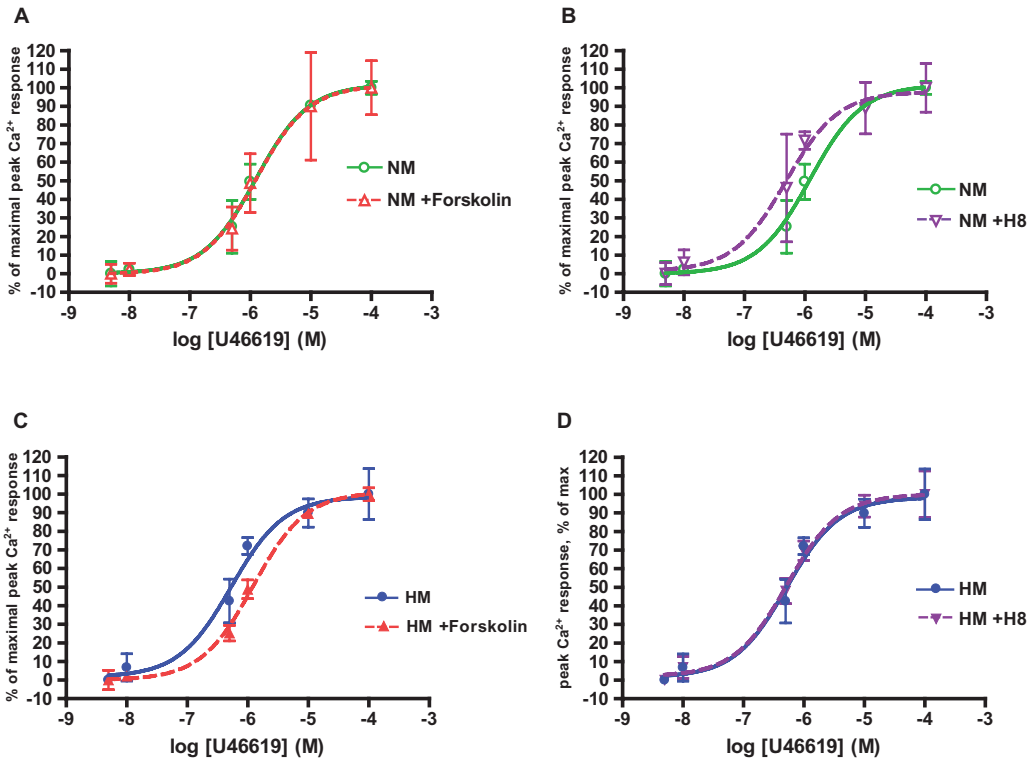

Figure 4

PKA activation state determines TP receptor sensitivity and EC_{50} of Ca^{2+} mobilization. Concentration response curves for the TP receptor agonist U46619, in pulmonary arterial myocytes pre-incubated with 1 μM forskolin (activating PKA) or H8 (inactivating PKA), or buffer, by fura-2AM. All Ca^{2+} values normalized as % of maximal concentration. Curves presented as mean ± SD. (A) Forskolin had no effect on the dose–response curve to U46619 in normoxic myocytes (NM). (B) H8 shifts the curve in NM to the left to resemble that of hypoxic myocytes (HM). (C) In HM, forskolin shifts the dose–response curve to the right to resemble that in NM. (D) H8 has no effect on the dose–response curve to the TP receptor agonist in HM.

Table 1

Log EC_{50} and EC_{50} values for PKA-activated and -inhibited myocytes from normoxic (NM) or hypoxic (HM) cultures

	NM	NM + Forskolin	NM + H8	HM	HM + Forskolin	HM + H8
Log EC_{50} ± SEM	−5.916 ± 0.037	−5.901 ± 0.066	−6.313 ± 0.253	−6.272 ± 0.163	−5.910 ± 0.025	−6.294 ± 0.118
EC_{50} (μM)	1.2	1.3	0.49	0.53	1.2	0.51

The values of log EC_{50} and EC_{50} for each group show differences between PKA-active (with forskolin) and PKA-inhibited (with H8) groups ($P < 0.0005$, $n = 15$). PKA activation decreased EC_{50} values approximately 2.5-fold.

hypoxic and normoxic conditions. Challenge of normoxic myocytes with iloprost stimulated significant cAMP generation at 1 min after challenge ($P < 0.01$), with return to baseline by 5 min. Hypoxic myocytes had a lower basal cAMP level ($P < 0.01$) and exhibited no measurable rise in cAMP after iloprost stimulation (Figure 6A). IP receptor protein expression was comparable in hypoxic and normoxic myocytes (Figure 6B). Decreased cAMP in hypoxic myocytes was not explained by increased breakdown, as cAMP-specific PDE activity in HM was diminished at all time points measured (Figure 6C, $P < 0.05$). Fractionation of measured cAMP-specific PDE activities indicated that the primary PDE isoforms in pulmonary arterial myocytes are PDE-3 and PDE-4, inhibited by rolipram and milrinone, respectively (Figure 6D). Scatchard analysis of saturation binding of ^{3}H-iloprost shows the K_d for this IP receptor agonist was elevated in hypoxic myocytes, suggesting that it is impaired IP receptor ligand affinity in hypoxia that induced the decreased cAMP signal (Figure 6E, F). The K_d of IP receptors was further increased (desensitized) by pre-treatment with iloprost, a known effect of this agonist. Stimulation of TP receptors with U46619 had no effect on the K_d value for the IP receptor ligand in hypoxic or normoxic myocytes, indicating the absence of receptor cross-regulation.

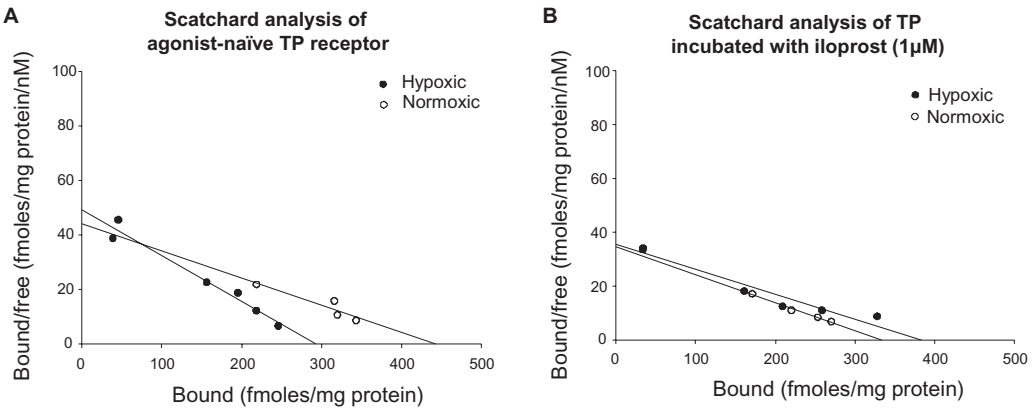

Figure 5

Ligand affinity of TP receptors in hypoxic myocytes can be normalized by incubation with iloprost. (A) Representative Scatchard plot for TP receptors in cell membrane fractions from hypoxic myocytes (HM) and normoxic myocytes (NM), using the radiolabelled TP receptor antagonist ^{3}H-SQ29548. Dissociation constant (K_d) is lower in HM than NM ($P < 0.01$; data represent three separate experiments). (B) Representative Scatchard plot for TP receptors. Pre-incubation of membrane fractions with iloprost (1 μM) increased K_d in both NM and HM membranes, eliminating the difference between these groups (P = ns; represents three separate experiments).

Table 2

TP receptor ligand association kinetics

	B_{max}	K_d
TP		
Normoxic	412 ± 7.2	8.8 ± 0.21
Hypoxic	315 ± 7.6	6.55 ± 0.05**
TP + Iloprost		
Normoxic	342 + 6.0	11.43 + 0.28
Hypoxic	361 + 9.4	10.29 + 0.28

Mean kinetics for TP receptor ligand association in hypoxic and normoxic myocyte cell membrane fractions, using the radiolabelled TP receptor antagonist ^{3}H-SQ29548. Dissociation constant (K_d) was lower in hypoxic than in normoxic myocytes (**$P < 0.01$; data represent three separate experiments). Pre-incubation of membrane fractions with 1 μM iloprost increased K_d of both hypoxic and normoxic cells, eliminating the difference between the groups (P = ns; data from three separate experiments).

Iloprost cannot normalize IP_3 or Ca^{2+} responses to TP receptor agonist in hypoxic myocytes

Generation of second messenger IP_3 was higher in basal and U46619-stimulated hypoxic myocytes than in normoxic myocytes. Pre-treatment with 1 μM iloprost did not attenuate agonist-mediated IP_3 release in hypoxic myocytes; however, the cAMP analogue, 8-bromo-cAMP did reduce IP_3 release in these cells (Figure 7A). Calcium responses to serial concentrations of U46619 were elevated in hypoxic myocytes compared with those in normoxic myocytes (Figure 7B, C) and were not normalized by iloprost pre-treatment.

Effect of milrinone on TP receptor–mediated Ca^{2+} mobilization

Hypoxic and normoxic myocytes loaded with fura-2AM were pre-incubated with 50 nM milrinone or vehicle, then stimulated with serial concentrations of the thromboxane analogue U46619. Peak calcium mobilization responses were quantified to derive normalized dose–response curves for each experimental condition. Milrinone had no effect on the TP receptor calcium dose–response curve in normoxic myocytes (Figure 8A, B), but it did shift the dose–response curve in hypoxic myocytes markedly to the right. The EC_{50} for U46619 in hypoxic myocytes treated with milrinone was completely normalized and comparable with previously stated EC_{50} values for TP receptors in PKA-active myocytes (Figure 8C, D; $P < 0.005$).

Effects of EP receptor or adenosine A_{2B} receptor stimulation on TP receptor–mediated calcium mobilization

To determine whether the attenuation of IP receptor–mediated regulation of TP receptors represented a more generalized derangement of cAMP generation, or a defect peculiar to the IP receptor, we examined the effects of two other pulmonary arterial $G\alpha_s$-coupled vasodilator receptors: prostaglandin EP receptors and adenosine A_{2B} receptors. Stimulation of EP receptors, with 11-deoxy prostaglandin E_1, normalized the dose–response curve to U46619 in hypoxic myocytes (Figure 9B; EC_{50} 1.2 μM, compared to 0.45 μM in untreated hypoxic myocytes). Stimulation of adenosine A_{2B} receptors with NECA, shifted the dose–response curves to U46619 further to the right in both normoxic myocytes (EC_{50} 18.3 μM) and hypoxic myocytes (EC_{50}, 13 μM); (Figure 9C).

Discussion

We previously reported sensitization of the response to U46619 in pulmonary arterial myocytes exposed to hypoxia

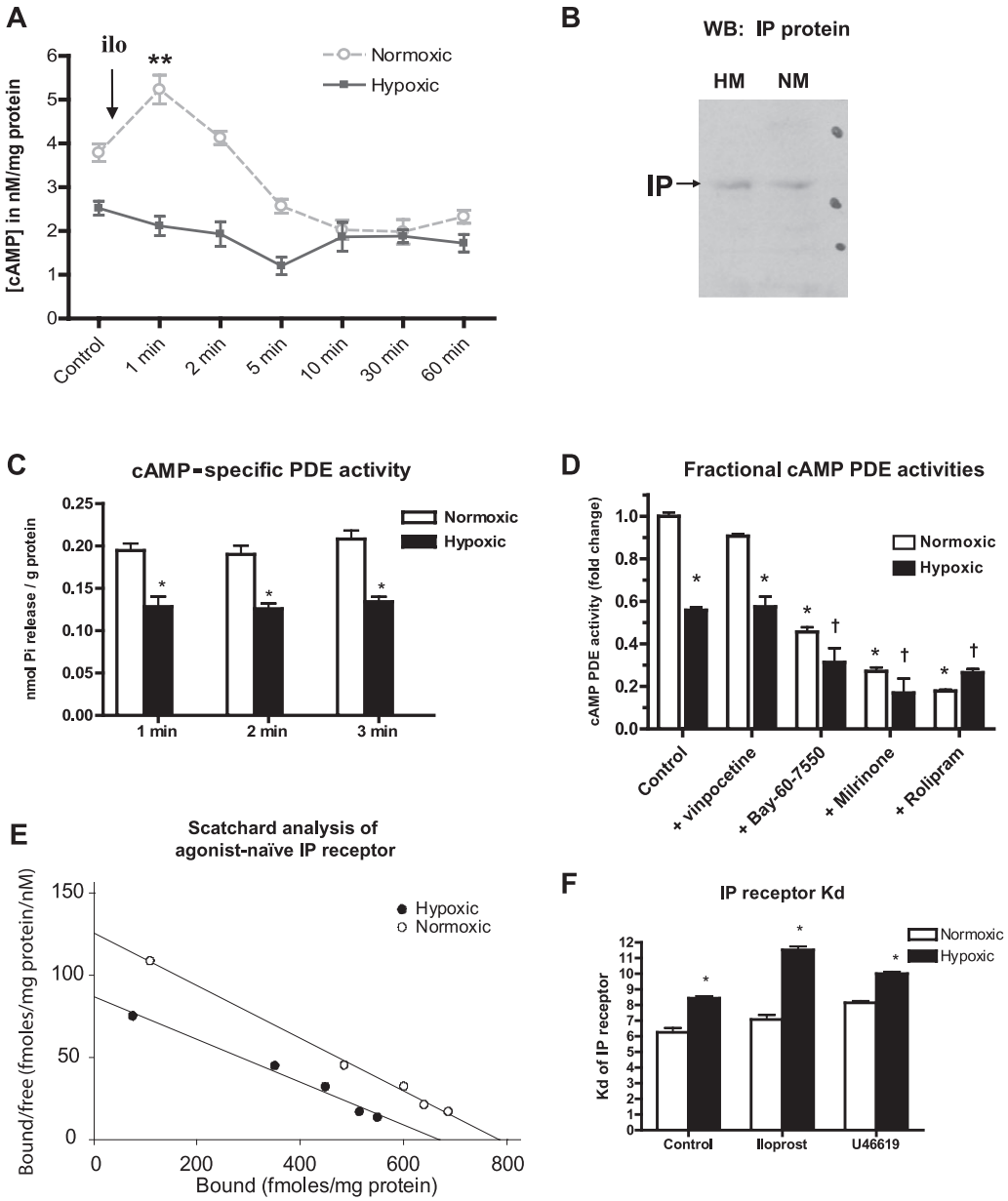

Figure 6

Attenuated IP receptor signalling in hypoxic myocytes is associated with decreased receptor affinity. (A) Stimulation of hypoxic (HM) and normoxic myocytes (NM) with the IP receptor agonist iloprost (ilo) results in cAMP release from NM (mean ± SEM; $P < 0.01$) but no measurable cAMP release from HM ($n = 4$). (B) Western blot of NM and HM lysates probed with polyclonal antibody to IP receptors (IP). (C) cAMP-specific PDE activity (as phosphate release from PDE substrate, mean ± SEM) is lower in HM ($P < 0.05$) compared with NM at all time points. (D) cAMP-specific PDE activity in NM and HM, fractionated by selective inhibition of PDE-1 (vinpocetine, 1 μM), PDE-2 (Bay-60–7550, 1 μM), PDE-3 (milrinone, 1 μM) or PDE-4 (rolipram, 1 μM). Mean ± SEM; $n = 6$; *$P < 0.05$ compared with normoxic control; †$P < 0.05$ compared with hypoxic control. (E) Representative Scatchard plot for IP receptors from agonist-naïve HM and NM cell membrane fractions, using ^{3}H-iloprost. (F) Dissociation constant (K_d in nM) of IP receptors is higher in HM, indicating impaired ligand affinity of IP receptors induced by hypoxia (mean ± SEM; $P < 0.05$; $n = 3$ separate experiments). Addition of iloprost further increases K_d (decreases affinity) in HM. Challenge with the TP receptor agonist U46619 has no effect on K_d of IP receptors.

and in myocytes derived from swine with hypoxic PPHN (Hinton *et al.*, 2006); this sensitization was associated with an increase in TP receptor phosphorylation (Hinton *et al.*, 2007). From the present study, we conclude that: (i) TP receptor ligand affinity and TP receptor–mediated signalling were increased by hypoxic exposure, resulting in displacement of the agonist dose–response curve to the left and upwards; (ii) sensitivity of TP receptors was regulated by the state of PKA activation; (iii) TP receptor activity could be normalized by raising cAMP, but not by direct stimulation of hypoxic myo-

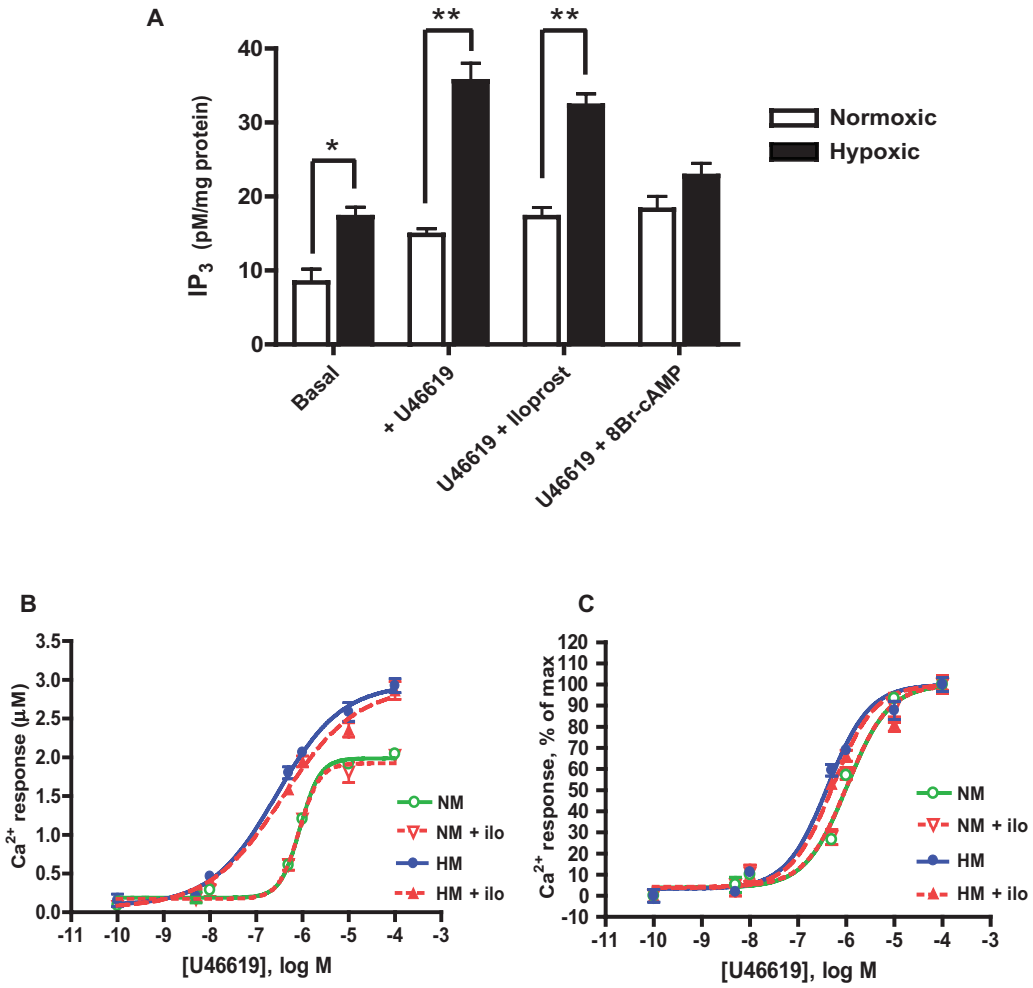

Figure 7

IP receptor stimulation cannot normalize IP_3 or Ca^{2+} responses to TP receptor stimulation in hypoxic myocytes. Hypoxic (HM) and normoxic (NM) myocytes were pre-incubated with 1 µM iloprost or 1 µM 8Br-cAMP prior to U46619 challenge. (A) Second messenger IP_3 was higher in basal and U46619-stimulated HM compared with NM (mean ± SEM; *$P < 0.01$, **$P < 0.001$). Pre-treatment with the cAMP analogue 8Br-cAMP, but not with iloprost, attenuated IP_3 production in HM ($P < 0.001$, $n = 3$). (B) In fura-2AM loaded myocytes, iloprost (ilo) pre-treatment had no effect on U46619-Ca^{2+} dose–response curves in NM or HM and (C) did not alter normalized dose–response curves (data in curves presented as mean ± SD; $n = 16$).

cytes with an IP receptor agonist; (iv) loss of regulation of TP receptors is due in part to IP receptor desensitization, which leads to diminished IP receptor signalling capability in hypoxic myocytes; and finally; and (v) TP receptors in hypoxic myocytes can be effectively desensitized by the PDE inhibitor milrinone (Figure 10).

An imbalance between production of TxA_2 and PGI_2 is implied in the pathogenesis of pulmonary hypertension accompanying hypoxic respiratory failure (Christman *et al.*, 1992) and septic pulmonary hypertension (Hammerman *et al.*, 1988; Ermert *et al.*, 2003). Infants with PPHN have markedly higher serum ratios of TxA_2 metabolite to PGE_2, indicating a predominance of vasoconstrictor eicosanoid activity (Sood *et al.*, 2007). Pulmonary arterial pressure correlates strongly with plasma TxA_2 (Bui *et al.*, 1991a; Bui *et al.*, 1992); increases in TxA_2 correspond with worsening postductal arterial hypoxemia (Nakayama *et al.*, 1992), while successful treatment of severe PPHN with extracorporeal oxygenation normalizes elevated plasma TxA_2 (Bui *et al.*, 1991b). TxA_2 underlies the early development of pulmonary arterial constriction in chronic hypoxia (Fike *et al.*, 2002). Both TxA_2 inhibition (Fike *et al.*, 2005) and TP receptor blockade (Cathcart *et al.*, 2008) can decrease hypoxic pulmonary hypertension, suggesting that prostanoids are crucial in the pulmonary vascular response to hypoxia.

Our data indicate that hypoxia decreases myocyte sensitivity to PGI_2, resulting in a loss of regulatory crosstalk and attenuated intrinsic inhibition of TP receptors. This increases the ability of TP receptors to bind agonist, leading to increased pro-contractile signalling. In hypoxic myocytes, both TP receptor sensitivity (EC_{50} for U46619) and reactivity (maximal Ca^{2+} response at receptor saturation) are enhanced. Both aberrations are corrected by forskolin and milrinone (which increase PKA activity). Thromboxane A_2, of which U46619 is a mimetic, has an exceedingly short half-life, and its serum concentration varies over a large range. The

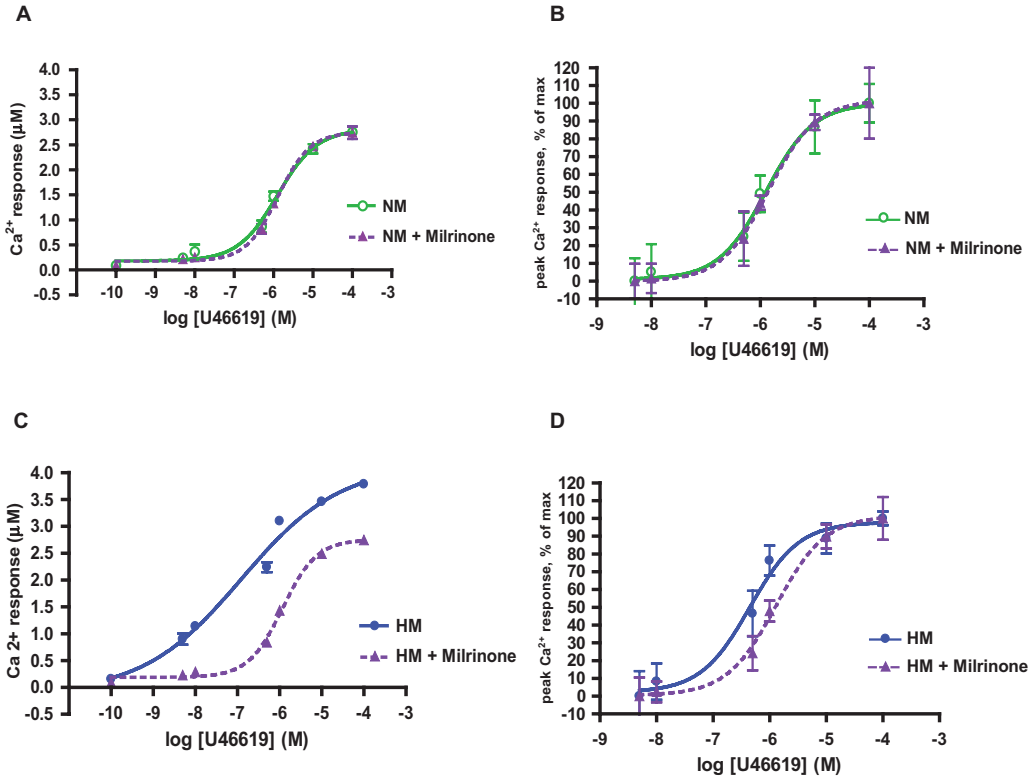

Figure 8

Both hypersensitivity and hyperreactivity of the TP receptors in hypoxic myocytes are normalized by exposure to milrinone. Hypoxic (HM) and normoxic (NM) myocytes loaded with fura-2AM were pre-incubated with 50 nM milrinone or diluent, then stimulated with serial concentrations of U46619 to derive concentration response curves. All curves presented as mean ± SD. (A) Milrinone had no effect on the maximal U46619 response in NM and (B) did not alter receptor sensitivity (NM EC_{50} 1.3 µM, NM + milrinone EC_{50} 1.4 µM, P = ns). (C) Milrinone attenuated the maximal Ca^{2+} response of HM to U46619 stimulation, and (D) decreased the sensitivity of the U46619 response curve (HM EC_{50} 0.45 µM; HM + milrinone EC_{50} 1.3 µM, $P < 0.005$; $n = 6$).

observed degree of sensitization would significantly increase TP receptor occupancy and, in context of fluctuating blood levels of agonist, result in greater time above the response threshold. In studies of the actions of milrinone in samples of radial artery, a 3.8-fold increase in the EC_{50} of the α-adrenoceptor agonist phenylephrine was considered clinically very significant (He and Yang, 2000).

TP receptors are phosphorylated at C-terminal serine residues through the action of the pulmonary vasodilator prostanoid PGI_2, influencing TP receptor sensitivity (Reid and Kinsella, 2003; O'Meara and Kinsella, 2004). PGI_2 activates the IP receptor coupled to adenylyl cyclase (Vane and Botting, 1995). IP–TP receptor interactions, through heterodimerization and regulatory crosstalk, generate both positive and negative feedback balancing their respective signals (Gleim *et al.*, 2009); TP receptor desensitization induced by IP receptor activation is primarily attributed to PKA (Walsh and Kinsella, 2000). We now report that TP receptor regulation via PKA, activated by the IP receptor, tightly controlled the dose–response relationship of TP receptor ligands and that the IP receptor was desensitized (decreased ligand K_d) in hypoxic myocytes. Myocyte PKA activity is decreased during hypoxia. Loss of protective IP receptor signalling in hypoxic myocytes induced TP receptor dysregulation. We conclude that PKA activity was both necessary and sufficient for desensitization of TP receptors. PKA activation in hypoxic myocytes via adenylyl cyclase (but not via IP receptor stimulation) normalized the TP receptor dose–response curve, and inhibition of PKA in normoxic myocytes shifted the dose–-response curve to the left and upwards to resemble that in hypoxic myocytes.

Superimposition of TP receptor agonist response curves from all treatment groups reveals only two curve morphologies and two EC_{50} value ranges for this receptor, as determined by PKA activation state (i.e. characteristic phospho-TP or non-phospho-TP receptor curves). This finding implies single-point regulation of TP receptor affinity. Robust activation of PKA with 8-bromo-cAMP raises PKA activity in hypoxic and normoxic myocytes by just 25% above the untreated normoxic control. However, this level of activation corresponds to a rise in PKA-dependent phosphorylation sufficient to fully desensitize the TP receptor. PDE-3 inhibition had no additional activating effect on PKA nor any inhibitory effects on Ca^{2+} mobilization after TP receptor stimulation in normoxic myocytes, suggesting this may reach a phosphorylation maximum or default basal state, below which TP receptor activity cannot be further reduced by phosphorylation. Conversely, inhibition of PKA with H8 reduces the measured PKA activity to half its control level, falling below a threshold level of PKA activity which allows full activation of the TP

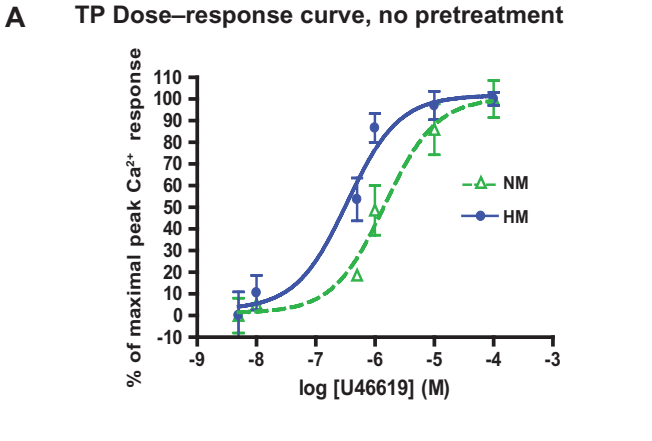

Figure 9

Hypersensitivity of TP receptors in hypoxic myocytes was normalized by activation of non- PGI_2 $G\alpha_s$-coupled receptors. (A) Dose–response curve (percent normalized, curves presented as mean ± SD) for agonist challenge of TP receptors, by fura-2 calciometry; $n = 18$. NM EC_{50}, 1.5 μM; HM EC_{50}, 0.34 μM. (B) TP receptor dose–response curve following 15 min pre-incubation of NM and HM myocytes with 1 μM 11-deoxy-prostaglandin E_1; HM EC_{50}, 1.3 μM. (C) TP dose–response curve following pre-incubation for 15 min with 1 μM NECA (NM EC_{50}, 18.3 μM, HM EC_{50}, 13 μM; $n = 16$–18).

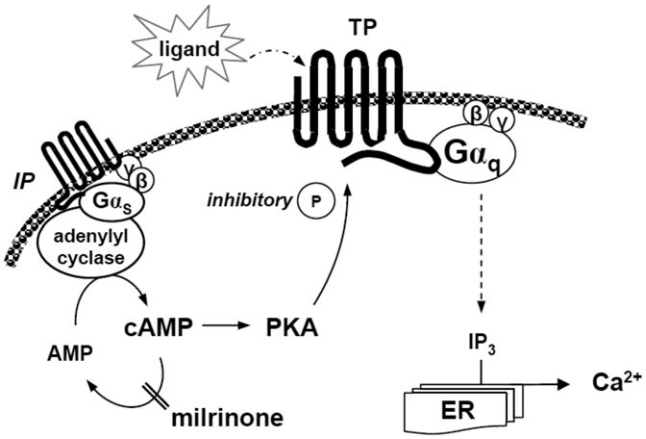

Figure 10

Proposed pathway of TP receptor regulation. We propose that milrinone down-regulates TP receptor affinity by restoring PKA activity in hypoxic myocytes, where IP receptor activity is impaired. ER, endoplasmic reticulum; IP, prostacyclin receptor; TP, thromboxane receptor; IP_3, inositol trisphosphate; P, phosphate group; PKA, protein kinase A.

receptor. In discussing the effects of PKA activation and inhibition, we note that the protein kinase inhibitor H8 used in these studies may be incompletely selective, with some inhibitory activity against PKG and PKC (at higher concentrations) as well as PKA (Chen *et al.*, 2000). However, while we have previously reported phosphorylation of TP receptors by more than one protein kinase, only PKA-mediated phosphorylation measurably altered TP receptor function (Hinton *et al.*, 2007). We have not quantified TP receptor phosphorylation in this study; however, we did fully describe TP receptor dephosphorylation after hypoxic exposure in an earlier study (Hinton *et al.*, 2007). We have thus limited the current study to analysis of upstream regulatory events.

Loss of pulmonary arterial IP receptor activity after prolonged agonist exposure is well described (Schermuly *et al.*, 2007). The rapid onset of IP receptor dysfunction in hypoxic myocytes is a new finding. Agonist exposure of the IP receptor is diminished under hypoxic conditions, as lowered PGI_2 and cAMP, as well as increased thromboxane levels, are reported in plasma and cerebrospinal fluid from hypoxic neonates (Liu *et al.*, 2003). While prolonged arterial remodelling in idiopathic pulmonary hypertension may decrease IP receptor expression (Lai *et al.*, 2008), such expression appears unaltered in hypoxic models of pulmonary hypertension (Abe *et al.*, 2001; Hoshikawa *et al.*, 2001). We also report unchanged IP receptor abundance following *in vitro* hypoxic exposure but a notable decrease in the activity of this receptor.

We describe two important effects of hypoxia on adenylyl cyclase–dependent signalling: (i) lower basal cAMP and PKA activity; and (ii) an attenuated cAMP response to IP receptor stimulation, due to decreased IP receptor–ligand interaction and/or receptor uncoupling. Hypoxia-induced defects in IP receptor–$G\alpha_s$ association and signalling, particularly attenuated receptor–ligand affinity, may cause the loss of IP receptor signal. Our data indicate that activation of the cAMP pathway downstream to the IP receptor (via direct adenylyl cyclase activation by forskolin, or via a stable cAMP analogue) can effectively regulate the TP receptor in hypoxic myocytes, while IP stimulation is ineffective. The decreased

cAMP elicited in hypoxic myocytes results from loss of $G\alpha_s$-linked adenylyl cyclase activation rather than accelerated cAMP degradation, as both resting and stimulated cAMP-specific PDE activity were lower in hypoxic cells. This may appropriately reflect lower basal and/or stimulated cAMP abundance (Tilley and Maurice, 2002). Inhibition of cAMP-specific PDEs increases cAMP, leading to relaxation of arterial and venous smooth muscle via decreased intracellular calcium. In models of pulmonary hypertension, PDE-3 expression is up-regulated and pulmonary artery relaxation enhanced by milrinone (Wagner *et al.*, 1997), while the inhibition of PDE-4 reverses hypertensive pulmonary arterial remodelling (Izikki *et al.*, 2009). In our studies, replacement of PKA activity, either using a cAMP analogue or by PDE-3 inhibition, down-regulates the sensitivity of hypoxic pulmonary arterial myocytes to a TP receptor agonist and restores their normal agonist dose–response curve. Additionally, we report normalization of the TP receptor dose–response relationship following pre-treatment with 11-deoxy-PGE_1, a prostanoid EP receptor agonist; and with adenosine-NECA, an adenosine A_{2B} receptor agonist. The ready ability of these unrelated $G\alpha_s$-coupled receptors to influence hypoxic TP receptor sensitivity suggests that cAMP generation by receptors other than IP receptors is preserved in hypoxic myocytes. The diminished IP receptor affinity observed in hypoxia may at least partly explain loss of stimulated cAMP response. Selective desensitization of other prostanoid receptors has been reported in hypoxic pulmonary arterial myocytes (Millen *et al.*, 2006). Further study of prostanoid receptor coupling and hypoxic regulation of adenylyl cyclase is warranted.

The cAMP pathway has been explored for therapeutic use in nitric oxide-refractory pulmonary hypertension (Rimensberger *et al.*, 2001). Milrinone is a PDE-3 inhibitor and pulmonary artery relaxant, which improves hypoxic pulmonary haemodynamics (Joynt *et al.*, 2008). Its mechanism of action is distinct from that of the well-described PDE-5 inhibitor sildenafil (Zhao *et al.*, 2001; Kirsch *et al.*, 2008). Pulmonary hypertension induced by TP receptor stimulation was alleviated by milrinone, with concurrent improvement of systemic haemodynamics (Gelvez *et al.*, 2004; Lobato *et al.*, 2006). The combination of milrinone and nitric oxide produced greater pulmonary relaxation than either drug alone (Holzmann *et al.*, 2001; Khazin *et al.*, 2004). There is considerable clinical interest in the pulmonary vasodilator effect of milrinone in neonates, as successful use of milrinone has been reported in small cohorts of infants with severe PPHN, refractory to nitric oxide (Bassler *et al.*, 2006; McNamara *et al.*, 2006). The milrinone concentration used in this study to attenuate TP receptor activity was 50 nM; higher doses completely ablated TP receptor responses (data not shown). This concentration elicits near maximal biochemical effect in cardiomyocytes *in vitro* (Mylotte *et al.*, 1985). Milrinone has direct vasodilator effects at concentrations above 10 nM (Liu *et al.*, 1997), with an EC_{50} for relaxation of pulmonary arterioles of 100 nM (Jhaveri *et al.*, 2004); its IC_{50} for selective PDE-3 inhibition is reported to be over a range from 490 nM (Zhao *et al.*, 2007) to as low as 56 nM (Tang *et al.*, 1994). We therefore consider that the observed effect of milrinone on TP receptor responsiveness in pulmonary arterial myocytes may occur *in vivo* at pharmacological concentrations of milrinone.

In view of the known effects of milrinone in attenuating inflammatory lung injury (Bueltmann *et al.*, 2009) and enhancing relaxation PGI_2 (Lakshminrusimha *et al.*, 2009), it is possible that the functional target of milrinone in pulmonary arterial myocytes may involve its interaction with prostanoid receptors. Our data provide the first clear evidence that milrinone may exert its biological effect, at least in part, by ameliorating TP receptor sensitization in hypoxic myocytes (Figure 10).

We conclude that hypoxia influences the balance of signalling in pulmonary arterial prostanoid receptors, favouring vasoconstriction mediated by TP receptors. PDE inhibition may attenuate these effects. In meconium aspiration and sepsis, both PPHN aetiologies marked by thromboxane signalling, treatment strategies that include milrinone may deserve further investigation.

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Conflicts of interest

None.

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