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Hypoxia increases pulmonary arterial thromboxane receptor internalization independent of receptor sensitization

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ABSTRACT

Persistent Pulmonary Hypertension of the Newborn (PPHN) is characterized by sustained vasospasm and an increased thromboxane:prostacyclin ratio. Thromboxane (TP) receptors signal via Gαq to mobilize IP_3 and Ca^{2+}, causing pulmonary arterial constriction. We have previously reported increased TP internalization in hypoxic pulmonary arterial (PA) myocytes.

Serum-deprived PA myocytes were grown in normoxia (NM) or hypoxia (HM) for 72 h. TP localization was visualized in agonist-naïve and -challenged NM and HM by immunocytochemistry. Pathways for agonist-induced TP receptor internalization were determined by inhibiting caveolin- or clathrin-mediated endocytosis, and caveolar fractionation. Roles of actin and tubulin in TP receptor internalization were assessed using inhibitors of tubulin, actin-stabilizing or -destabilizing agents. PKA, PKC or GRK activation and inhibition were used to determine the kinase responsible for post-agonist receptor internalization.

Agonist-naïve HM had decreased cell surface TP, and greater TP internalization after agonist challenge. TP protein did not sort with caveolin-rich fractions. Inhibition of clathrin prevented TP internalization. Both actin-stabilizing and -destabilizing agents prevented TP endocytosis in NM, while normalizing TP internalization in HM. Velocity of TP internalization was unaffected by PKA activity, but PKC activation normalized TP receptor internalization in HM. GRK inhibition had no effect.

We conclude that in hypoxic myocytes, TP is internalized faster and to a greater extent than in normoxic controls. Internalization of the agonist-challenged TP requires clathrin, dynamic actin and is sensitive to PKC activity. TP receptor trafficking and signaling in hypoxia are pivotal to understanding increased vasoconstrictor sensitivity.

1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is characterized by increased pulmonary vascular resistance, due in part to narrowing of pulmonary arteries [1], muscularization of distal pulmonary arteries and decreased activity of endothelial nitric oxide synthase [2]. PPHN has an incidence up to 6.8 in 1000 live births [3] due to perinatal diseases including sepsis, meconium aspiration or perinatal hypoxia [4].

Eicosanoids and their receptors are known to be key mediators in the pathophysiology of PPHN. A decreased prostacyclin (PGI_2):-thromboxane (TxA_2) ratio is reported in a hypoxic model of PPHN [5], favoring the smooth muscle vasoconstrictor thromboxane. Among eicosanoid receptors, the thromboxane prostanoid receptor (TP) is the major regulator of vascular resistance in the pulmonary circuit. Activation of this G protein coupled receptor (GPCR) by thromboxane (TxA_2) or a mimetic agonist (U46619), results in signaling through Gαq to generate IP_3 [6], mobilization of intracellular Ca^{2+}, and smooth muscle contraction [7]. TP also activates the Rho kinase pathway, modulating myosin phosphorylation [8], and is linked to polymerization of actin filaments [9], increasing contractile force.

TP receptor-ligand affinity and receptor internalization is regulated by phosphorylation of specific serines and threonines within

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the receptor's intracellular loops or C-terminal tail [10]. Serine/threonine kinases such as protein kinase A (PKA), protein kinase C (PKC) and G protein related kinases (GRK) are most commonly associated with GPCR desensitization [11,12]. Following phosphorylation of the agonist-activated GPCR, receptor desensitization continues through β-arrestin binding [13], which can temporarily shut off the signal by interfering with receptor - G protein binding [14], or abrogate the signal entirely via receptor internalization [15]. Endocytosis of GPCRs can be mediated by clathrin- or caveolin-coated vesicles [16], and requires a functional actin cytoskeleton [17]. Receptor internalization normally occurs following agonist exposure, but it is not exclusive to desensitization [18]. After internalization, GPCRs can be recycled back to the plasma membrane or sorted for lysosomal degradation [19].

We have previously demonstrated that TP signaling is altered following *in vivo* and *in vitro* hypoxic treatment of neonatal porcine pulmonary arterial smooth muscle, in a well-established model of PPHN [20]. Regulatory serine phosphorylation of TP is markedly decreased after hypoxic exposure. This results in a hypersensitive and hyper-reactive TP receptor that heightens Ca^{2+} mobilization from the sarcoplasmic reticulum [20], and significantly promotes polymerization of actin [9]. Hypersensitivity of hypoxic TP receptor reflects its decreased Kd (increased receptor agonist affinity), which is normalized by incubation with protein kinase A; TP Bmax (open receptor abundance) is not increased by hypoxia [21]. In chronically hypoxic pulmonary arterial myocytes, cell surface TP abundance markedly decreases [20]. In most vascular GPCRs, including adrenergic receptors, receptor-mediated activity is upregulated via an increase in Bmax [22], while receptor internalization typically functions as a negative feedback mechanism [23]. TP receptor internalization in hypoxic myocytes may be understood as a regulatory negative feedback to hypoxia-induced hypersensitivity; however the mechanism of receptor cycling in this context remains to be elucidated.

This study examines mechanisms of hypoxic TP receptor internalization by examining cell surface and cytosolic receptor abundance, evaluating the involvement of clathrin and caveolin in U46619-mediated receptor endocytosis, and the role of actin in internalization of the hypoxic TP receptor. We hypothesized that hypoxia increases the rate and extent of post-agonist internalization of TP, mediated by clathrin; and that the increased internalization of the TP receptor in hypoxia is partly due to an increase in polymerized actin in hypoxic pulmonary arterial myocytes. Finally, we identify the serine/threonine kinase (PKA, PKC or GRK) responsible for initiating internalization of the ligand-bound TP receptor. Since we have previously shown that activation of PKA causes desensitization and downregulation of TP signaling [21], we hypothesized that phosphorylation of TP by protein kinase A would diminish hypoxic TP internalization.

2. Methods

The University of Manitoba Central Animal Care approved animal care protocols, in accordance with the Canadian Council of Animal Care and US National Institutes of Health guidelines.

2.1. Cell culture and in vitro hypoxia

Pulmonary arterial smooth muscle cells (PASMC) were obtained from newborn piglets (<24 h old) using a dispersed cell culture method described previously [24]. Briefly, 2nd to 6th generation pulmonary arteries were microdissected and 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. Arteries were washed with Ca^{2+}-reduced (20 μM $CaCl_2$) HBS solution, minced, then enzymatically digested with type I collagenase (1750 U/mL), and papain (9.5 U/mL) in Ca^{2+}-reduced HBS with 1 mM dithiothreitol and 2 mg/mL bovine serum albumin (BSA), for 15 min at 37 °C. The dispersed cells were centrifuged at 1200 rpm for 5 min, and washed with Ca^{2+}-free HBS solution. PASMC were plated in Ham's F-12 medium containing L-glutamine, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at density 4.4×10^4 cells/cm^2. Confluent myocytes were serum deprived for 2 days in F-12 media containing 1% penicillin/streptomycin and 1% insulin-transferrin-selenium to synchronize in a contractile phenotype. Serum deprived PASMC were then randomized to hypoxia (HM, 10% O_2; obtained by N_2 washout of a sealed plexiglass chamber) or to normoxia (NM, 21% O_2 + 5% CO_2) for an additional 3 days. Protocol for *in vitro* hypoxic exposure in Fig. 1.

2.2. TP receptor localization by immunofluorescence

Confluent serum-deprived PASMC in hypoxic and normoxic groups were challenged with 1 μM U46619 (TP receptor agonist; Sigma), 10 μM SQ29548 (TP receptor antagonist; Sigma) or diluent for 2 h in media. Cells to be analyzed for membrane receptor abundance were washed twice with PBS, and then fixed with ice-cold methanol (80%) for 15 min at −20 °C. Cells for whole cell receptor abundance were rinsed with cold CB buffer (in mM: 10 MES, 150 NaCl, 5 EGTA, 5 $MgCl_2$, 5 glucose), fixed with 3% paraformaldehyde (15 min), and then permeabilized with 3% paraformaldehyde + 0.3% Triton X-100 (5 min). Non-specific binding was blocked by incubation in 1% BSA + Cyto-TBS for 20 min at room temperature. Cells were then incubated overnight at 4 °C in rabbit anti-TP receptor antibody (1:10; Cayman Chemicals), followed by incubation with FITC-conjugated donkey anti-rabbit secondary (1:25; Jackson ImmunoResearch) for 2 h at room temperature. Coverslips were mounted and visualized by CCD fluorescence microscopy. Digital images were captured under standardized conditions to quantify membrane-to-total TP localization using Image-Pro software. Coverslips were stained with Texas Red Phalloidin (1:50; Life Technologies) to confirm absence of intracellular actin filament staining following intact cell membrane (methanol fixation) versus permeabilized membrane (PFA fixation), Supplemental Fig. 1.

2.3. TP receptor localization by membrane and cytosol fractionation

Contractile PASMC grown in hypoxic or normoxic conditions were incubated with 1 μM U46619 or diluent in media for 2 h, then lysed in ice-cold RIPA buffer (in mM: 20 MOPS, 2 EGTA, 5 EDTA, 30 sodium fluoride, 40 beta-glycerophosphate, 10 sodium

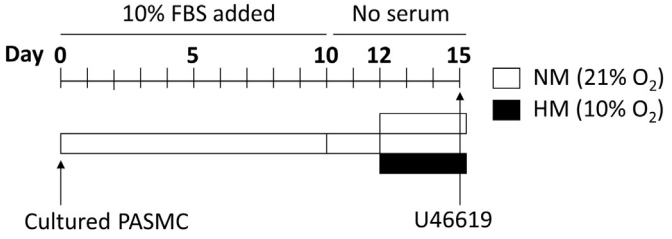

Fig. 1. In vitro hypoxia protocol for neonatal porcine PASMC. Primary porcine pulmonary arterial smooth muscle cells (PASMC) were grown to 75% confluence in fetal bovine serum (FBS) before synchronization in a contractile phenotype by serum deprivation. Myocytes were placed in normoxic (NM, 21% O_2) or hypoxic (HM, 10% O_2) environment for 72 h. Immediately prior to collection, myocytes were challenged with TP agonist U46619 (10^{-6} M) or diluent.