POST-TRANSLATIONAL MODIFICATIONS OF THROMBOXANE RECEPTOR G-PROTEIN ALPHA Q COMPLEX IN HYPOXIC PPHN

By

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CANADA

DISCLAIMER

This modified sandwich thesis comprises three multi-authored manuscripts, all of which contain a majority of original work contributed by the author of this thesis, but which also contain varying portions of work contributed by collaborating lab members. The Gαq palmitoylation and Thromboxane receptor (TP) phosphorylation studies in this thesis are published in American Journal of Respiratory Cell and Molecular Biology and British Journal of Pharmacology respectively; the adenylyl cyclase (AC) activity study is in currently preparation for publication. Individual author contributions are given below.

1. *Palmitoylation of Gαq determines its association with the thromboxane receptor in hypoxic pulmonary hypertension. Am J Respir Cell Mol Biol. 2014 Jan;50(1):135-43.*
   Anurag Singh Sikarwar is the first author of the Gαq palmitoylation study and was primarily responsible for study design, experimentation and data analysis. Within this manuscript, the ATP dose-response (in pulmonary artery smooth muscle cells) and U46619 dose-response in aortic myocytes was done with help from Martha Hinton.
   Santhosh Thomas performed the inositol 1,4,5 triphosphate assay. 14C-palmitate and 3H-leucine uptake was done by Anurag Singh Sikarwar with help from Santhosh Thomas. Force myography, Fura-2AM calcium imaging on pulmonary artery smooth muscle cells and all work on the HEK cell line was performed and analyzed by Anurag Singh Sikarwar.

2. *Thromboxane receptor hyper-responsiveness in hypoxic pulmonary hypertension requires serine 324. Br J Pharmacol. 2014 Feb;171(3):676-87.*
   Anurag Singh Sikarwar and Santhosh Thomas equally contributed as first authors of this study toward the study design, experimentation and data analysis. Anurag Singh Sikarwar and Santhosh Thomas designed thromboxane receptor α (TPα) serine mutants and performed the calcium assay experiments on stable and transiently transfected cells. Anurag Singh Sikarwar prepared the stable cell lines for human and porcine TPα overexpression, and carried out the force myography experiments. Santhosh Thomas performed radioligand binding studies, and protein kinase A and protein kinase C activity experiments. TP serine immunoprecipitation experiments were done by Martha Hinton.

3. *Adenylyl cyclase activity is impaired by pulmonary arterial hypoxia (unpublished)*
   Santhosh Thomas initially measured basal and stimulated AC activity, and completed the phosphodiesterase activity assays. Subsequent experimental design, data collection and data analysis were the responsibility of Anurag Singh Sikarwar. Anurag Singh Sikarwar did the force myography experiments, and performed AC activity assays following treatment with pertussis toxin and BAPTA. Anurag Singh Sikarwar and Martha Hinton jointly carried out the Western blot and immunoprecipitation experiments. Martha Hinton did the expression profile of AC enzyme isoforms.

Abstract

Introduction: Persistent pulmonary hypertension of the newborn (PPHN) is associated with an elevated thromboxane to prostacyclin ratio, pulmonary artery (PA) hyperreactivity and hypersensitivity. Thromboxane receptor (TP), coupling with G-protein Gαq causes pulmonary vasoconstriction; whereas prostacyclin receptor (IP), coupling with Gαs, causes vasodilation and TP phosphorylation via adenylyl cyclase (AC)-cAMP-protein kinase A (PKA), desensitizes TP. Both TP phosphorylation and Gαq palmitoylation play major roles in regulation of signaling through the TP-Gαq complex. We hypothesized that increased Gαq palmitoylation and decreased AC activity could cause hypoxic TP hyperresponsiveness. We studied the impact of hypoxia on selected post-translational modifications of the receptor-G-protein complex, determining TP vasoconstriction: Gαq palmitoylation, TP phosphorylation and upstream AC activity.

Methods: Force responses to thromboxane mimetic U46619, palmitoylation inhibition by 2-bromopalmitate (2-BP) and AC activation (forskolin) were studied by myography in hypoxic PPHN and control newborn swine pulmonary artery. Ca^{2+} mobilization was studied by fluorescent calcium indicators fura-2AM in pulmonary myocytes (PASMC), and fluo-4NW in HEK293 cells. Effects of hypoxia on Gαq palmitoylation were studied by metabolic labeling. Gαq cysteines and TP serines were mutated to determine sites of post-translational modifications. Protein expression and receptor-G-protein coupling were studied by Western blot and co-immunoprecipitation. PKA activity was assayed; and AC activity quantified.

Results: Hypoxia increases Gαq palmitoylation, without increasing total palmitate uptake. Palmitoylation inhibition decreases U46619-stimulated force generation as well as Ca^{2+}

mobilization in PPHN PA rings and hypoxic PASMC. Mutation of palmitoylable cysteine and palmitoylation inhibition proportionately decrease U46619-mediated Ca^{2+} mobilization in HEK293 cells. TP serine phosphorylation is decreased by hypoxia due to decreased PKA activity; this causes TP hypersensitivity and hyper-reactivity. Serine 324 of TPα is the target of PKA-mediated desensitization. AC activator-induced relaxation is reduced in PPHN PA. Basal and receptor-stimulated AC activity are decreased in hypoxic PASMC. Decreased AC activity is not due to decreased AC expression, ATP availability nor increased Gαi activation. IP coupling with Gαs is increased in hypoxia.

Conclusion: Increased Gαq palmitoylation plays a role in TPα hyper-responsiveness in hypoxic PPHN. Hypoxia also reduces responses to agents acting through AC, unleashing TP-mediated vasoconstriction. Reactivation of pulmonary AC might be useful therapeutically to promote vasodilation and TP desensitization.

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Anurag Singh Sikarwar

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List of abbreviations

α	alpha
β	beta
γ	gamma
μM	micro mol
A	Alanine
AC	adenylyl cyclase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
2-BP	2-bromopalmitate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
BIS	bisindolylmaleimide I
Bmax	maximum binding
BSA	bovine serum albumin
Ca^{2+}	calcium
$CaCl_2$	calcium chloride

CaM	calmodulin
cAMP	3’-5’-cyclic adenosine monophosphate
cGMP	3’-5’-cyclic guanosine monophosphate
Cl^-	chloride
COX	cyclooxygenase
CPI-17	protein kinase C-potentiated myosine phosphatase inhibitor
C-terminus	carboxyl-terminus
Cys	cysteine
DAG	diacylglycerol
ECMO	extra corporeal membrane oxygenation
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ET_A	endothelin-1 receptor (type A)
ET_B	endothelin-1 receptor (type B)
FBS	fetal bovine serum
FiO_2	fraction of inspired oxygen

GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine 5-diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(gamma-thio)triphosphate
h	hour/s
H8	N-(2-(methylamino)ethyl)isoquinoline-5-sulphonamide dihydrochloride
H_2O	water
HBS	HEPES buffered saline solution
HEK	human embryonic kidney cell
I-BOP	(1S-(1α,2α(Z),3β(1E,3S*),4α))-7-(3-(3-hydroxy-4-(4-iodophenoxy)-1-butenyl)-7-oxabicyclo(2.2.1)hept-2-yl)-5-heptanoic acid
IP	prostacyclin receptor
IP_3	inositol triphosphate
ITS	insulin-transferrin-selenium
K^+	potassium

KCl	potassium chloride
Kd	dissociation constant
LARG	leukemia-associated RhoGEF
LIMK	LIM kinase
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MLC	myosine light chain
MLCK	myosine light chain kinase
MLCP	myosine light chain phosphatase
MYPT	myosine phosphatase targeting protein
mv	milli volt
N_2	nitrogen
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	sodium dihydrogen phosphate
NO	nitric oxide

NOS	nitric oxide synthase
N-terminus	amino terminus
O_2	oxygen
p-	phsopho-
PA	pulmonary artery
PASMC	pulmonary artery smooth muscle cell
hPASMC	human pulmonary artery smooth muscle cell
pPASMC	porcine pulmonary artery smooth muscle cell
PBS	phosphate buffered saline
PDE	phosphodiesterase
PGI_2	prostacyclin
Pi	inorganic phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C

PMA	phorbol 12-myristate 13-acetate
PO_2	partial pressure of oxygen
PPHN	persistant pulmonary hypertension of newborn
PS	penicillin-streptomycin
PTX	pertussis toxin
PVR	pulmonary vascular resistance
RGS	regulator of G-protein signaling
ROC	receptor-operated calcium channel
ROCK1/2	rho associated coiled-coil kinase ½
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RyR	ryanodine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
SOC	store-operated calcium channel
SQ29458	7-(3-((2-(phenylcarbamoyl)hydrazinyl)-7-oxabicyclo(2.2.1)heptan-2-yl)hept-5-enoic acid
STIM1	stromal interaction molecule 1

TASK-1	two-pore domain acid sensitive potassium channel-1
TBS	tris-buffered saline
TBST	tris-buffered saline containing tween
TM	transmembrane
TP	thromboxane prostanoid receptor
TRPC	transient receptor potential canonical channels
TxA_2	thromboxane A_2
U46619	9,11-dideoxy-9 alpha, 11 alpha-methanoepoxy prostaglandin F_2alpha
VDCC	voltage-dependent cation channel
Vmax	maximum velocity of shortening

1 Literature review

1.1 Incidence natural history and pathophysiology of PPHN

1.1.1 Fetal circulation

The placenta serves as an organ of gas exchange in the developing fetus, allowing fetal lungs to grow supported by bronchial circulation and in the absence of significant pulmonary perfusion, and be ready to take over the task of gas exchange at the time of birth [1]. Fetal umbilical arteries carry deoxygenated blood from the fetus to the low vascular resistance placenta; after gas exchange, oxygenated blood is returned to the fetus by the umbilical vein [2]. About 50-60% of oxygenated blood in the umbilical vein bypasses hepatic circulation via the ductus venosus and flows in a stream along the dorsal aspect of the inferior vena cava (IVC). At the junction of IVC and right atrium, the Eustachian valve directs the dorsally streaming oxygenated blood to the left atrium via foramen ovale. From the left atrium, blood enters into the left ventricle, which ejects oxygenated blood into the ascending aorta. Oxygen saturation of blood in fetal left atrium is 65%, down from 80-90% saturation in umbilical vein. This is due to some mixing with the desaturated blood in IVC coming from the lower body [3]. Blood from superior vena cava and the coronary sinus, along with ventral stream of desaturated systemic blood in IVC, enters the right ventricle via tricuspid valve. From right ventricle this desaturated blood is ejected into pulmonary artery which directs blood to descending aorta via the ductus arteriosus [4]. Only 8-10% of combined ventricular output enters into the fetal pulmonary circulation due to very high pulmonary vascular resistance (PVR) [5]. Recent phase contrast magnetic resonance imaging studies on near-term human fetuses suggest that lungs receive about 25% of right ventricular output via the pulmonary arteries; the rest (75%) bypasses the lungs and is directed towards the

ductus arteriosus [6]. High PVR in fetal lungs is due to several factors: liquid in air spaces raising extra luminal pressure on surrounding pulmonary arteries, the absence of an air liquid interface, low resting alveolar and arterial oxygen tension, and high muscle mass and resting tone of pulmonary arteries [7]. In growing lungs at the canalicular stage, PVR is high because of an immature pulmonary vascular bed with low cross-sectional area and small vascular capacity. During the early saccular stage there is a fall in PVR, and increase in lung blood flow, due to proliferating pulmonary vessels that are irresponsive to maternal hyperoxygenation [8]. By early preterm and late preterm gestation, PVR rises again despite the presence of more pulmonary vessels to accommodate blood flow, as by this time pulmonary circuit smooth muscle has developed fully and is under active vasoconstriction due to endothelin-1, thromboxane and hypoxemia. Maternal hyperoxygenation at this stage results in a fall in PVR with more flow, suggesting that pulmonary vessels have acquired vasoreactivity [8].

1.1.2 Lung development

Lung development is a complex process, which starts from the third week of gestation and runs up to 8 years of age [9]. A very brief description of the phases of lung development is given below. On the basis of histological appearance, lung development is divided into embryonic, pseudoglandular, canalicular, saccular and alveolar stages [10]. In the embryonic phase (3-7 weeks), respiratory diverticulum forms from the ventral wall of the foregut, that later bifurcates to give rise to right and left primary bronchial buds. Further rounds of branching give rise to secondary and tertiary bronchi on the right and left side [9, 11]. Arteries and veins also start to develop in coordination with airways at this time [12]. In the pseudoglandular phase (5-17 weeks), growing lungs have multiple epithelial tubules with increased rates of cell proliferation, finishing bronchial divisions by the end of the pseudoglandular stage. Major advances in this

stage are the appearance of cilia in proximal airways and the beginning of cartilage development [11], as well as the presence of pre-acinar vessels as a result of vasculogenesis, as the developing vessels use the airways as a template [10, 12]. Pulmonary vessels develop through the process of vasculogenesis and angiogenesis[13]. Primitive angioblasts and hemangioblasts differentiate to give new vessels (vasculogenesis) further sprouting and branching of preexisting vessels generates distal vessels (angiogenesis) [13]. The canalicular stage of lung development (16-26 weeks) is characterized by the growth of respiratory epithelium and formation of pulmonary acinus and respiratory bronchioles [14]. Branching of the arteries and veins continues in step with the airways [12]. Infants born by the end of this stage can survive due to the presence of respiratory epithelium and an adequate vascular bed [14]. By the end of the saccular stage (24-36 weeks), lungs are equipped with more generations of alveolar ducts, and ciliated cells are well developed in the conducting airways [9]. Maturation of type II epithelial cells causes an increase in surfactant synthesis. During the alveolar stage (36 weeks-8 years), terminal ducts and saccules further divide to give rise to alveolar ducts and alveoli. Maturation of the alveolar capillary membrane further increases the gas exchange surface area [9].

Lung development is different in the various animal species used to study newborn lung diseases. Rats and mice are born with saccular lungs [14] making them a good choice to study premature lung diseases such as BPD; whereas similar to humans [14], pigs and sheep are born with alveolar lungs. That is why animals such as pigs and sheep are more suited to study PPHN pathophysiology, as PPHN is a disease of the term (alveolar) lung.

1.1.3 Cardio-pulmonary events at the time of birth

Several factors work together to perform the complex and rapid task of normal circulatory transition during birth. Fetal airways are filled with low protein chloride rich liquid secreted by

airway epithelium [15]. This liquid keeps the lungs distended and acts as a stimulus for lung development [16]. Lung fluid clearance is essential to make space for breathing air during birth. Both production and volume of lung fluid decrease in fetal rabbits and sheep before birth [17]. A recent study using phase contrast X-ray imaging studies visualised air intake and airway liquid clearance at birth in newborn rabbits, and reported that irrespective of spontaneous and mechanical ventilation, inspiration and lung inflation is the primary driver of airway liquid clearance by increasing pressure across the airway wall which forces liquid from airway to surrounding tissue [18]. Other mechanisms suggest that during the birth increase in thyroid hormone, cortisol and catecholamines block the chloride-mediated secretion of fetal lung fluid and promote reabsorption of fluid by activating basal Na^+-K^+ ATPase [19]. Labor and catecholamines released at parturition induce surfactant secretion into lung alveoli [19, 20]. At the time of birth, crying and air breathing inflate lungs and stimulate dilatation and recruitment of intra-acinar pulmonary arteries, causing a rapid fall in PVR [21]. A recent study using phase contrast X-ray imaging and angiography on newborn rabbits reports that after birth, the increase in pulmonary blood flow is not related to ventilated lung regions [22]. Further fall in PVR is due to the release of vasodilators such as prostacyclin and nitric oxide and bradykinin from the endothelium as a result of increased pulmonary oxygen levels [23-25]. Removal of low vascular resistance placenta by clamping the umbilical cord raises systemic vascular resistance (SVR); a sudden drop in flow through the IVC causes a fall in venous return to the right atrium, but at the same time increased pulmonary blood flow compensates for umbilical venous return as the source of preload for the left ventricle [26]. Increased pulmonary blood flow reverses blood flow through ductus arteriosus and raises the left atrial pressure above right atrial pressure, which closes the foramen ovale [27].

1.1.4 Postnatal structural and functional changes in pulmonary artery

Studies from piglets suggest that postnatal pulmonary structural and functional changes involve three overlapping stages, namely: dilatation and recruitment, reduction in muscularity, and growth [28]. Dilatation and recruitment take place after 5 minutes of life and continues up to 24 hours of life in the non-muscular and partially muscular arteries. This results in decrease in PVR by 11 min after birth [21]. Non-muscular arteries have pericytes and swollen endothelial cells on elastic lamina; these vessels undergo flattening of endothelial cells to spread out in vessel wall via actin depolymerization. This process increases the arterial luminal diameter and lowers PVR up to 80% of fetal value [29]. In the second stage of reduction in muscularity, which runs from first 24 hours to 2 weeks, partially muscular arteries lose their smooth muscle cells, whereas arteries with complete smooth muscle cells revert to a partially muscular state. By two weeks of age, these changes in muscularity of arteries not only increases lumen diameter and decrease the PVR/SVR ratio, but also reduce the arterioles' capacity to constrict [21]. In the third stage, growth of pulmonary arteries occurs by vascular remodelling till an adult pattern is reached at six months of age [21].

1.1.5 Persistent pulmonary hypertension of newborn

Physiological mechanisms that contribute to high fetal PVR and right to left shunts that bypass blood from the lungs such as ductus arteriosus and foramen ovale must successfully terminate to promote fall in PVR and increased blood flow into the lungs to allow smooth transition of gas exchange function from the placenta to lungs. The failure of normal postnatal pulmonary transition maintains persistence of high PVR and right to left shunts after birth results in a lethal syndrome of term or near-term infants which is called persistent pulmonary hypertension of newborn (PPHN) [30-32]. PPHN has an incidence up to 1.9 (range 0.4-6.8) per 1000 live births

and has a mortality rate ranging 4 to 33 percent if not managed effectively [33]. Early PPHN is characterized by failure of pulmonary arterial relaxation causing supra-systemic pulmonary pressures and hypoxemia and presence of right-to-left shunting of blood across ductus arteriosus and foramen ovale [34]. Vasodilator therapy becomes less effective with disease progression and in the late stage (14-day) gives rise to pulmonary arteries with thickened media due to hypertrophy and hyperplasia of smooth muscle cells as well as extracellular matrix deposition [35, 36].

1.1.6 Aetiology of PPHN

1. Idiopathic PPHN

   PPHN in absence of pulmonary and cardiac disease is called idiopathic PPHN and has incidence up to 1.9/1000 live births [37]. Infants who die from idiopathic PPHN have pulmonary pressures above systemic pressures, hypoxemic due to the right to left shunting of blood across ductus arteriosus and foramen ovale, and extension of muscularization upto normally non muscularized intra-acinar arteries [38, 39]. Antenatal use of non-steroidal anti-inflammatory drugs has been shown to be associated with idiopathic PPHN [40].

2. PPHN secondary to other pulmonary diseases

   PPHN is mostly seen as secondary to many neonatal pulmonary disorders such as respiratory distress syndrome, meconium aspiration syndrome, congenital diaphragmatic hernia (CDH), bronchopulmonary dysplasia (BPD), pneumonia and sepsis [41, 42]. In meconium aspiration syndrome, perinatal aspiration of dark green sticky meconium in to fetal airways leads to airway obstruction; this severely interferes with post natal lung

inflation and the normal fall in PVR, by causing mechanical obstruction and triggering lung injury [43, 44] CDH is characterised by reduced and thickened pulmonary vascular bed with adventitial and medial thickening opposing postnatal drop in high PVR [45]. BPD is common in preterm infants whose lungs start out ventilated at the canalicular stage [46]. Pulmonary arteries in these infants are reduced in number, show abnormal vasoreactivity and distal muscularization and have thickened media [47]. All these anomalies maintain a PVR high at birth causing PPHN. According to the World Health Organization's Nice clinical classification, pulmonary arterial hypertension is divided into 5 categories. PPHN has been placed separately in subcategory in category 1 due to its specific nature, time of onset and therapeutic strategies used to manage the disease [48].

1.1.7 Animal models of PPHN

PPHN is a multi-factorial disease and can present itself alone or secondary to other cardio-pulmonary disease; so there are several models used to generate the disease in laboratory to reflect a particular disease phenotype. Broadly, animal models of PPHN are of two types: acute models and chronic models [49].

Acute models

I. **Neonatal sepsis**: Intravenous administration of live or heat-killed group B beta hemolytic streptococci into piglets and lambs trigger acute biphasic pulmonary hypertension with early and late phase [50, 51]. The early phase is mediated through thromboxane and causes acute pulmonary vasoconstriction, whereas late phase is associated with systemic hypotension and characterized by infiltration of

inflammatory cells into lung parenchyma causing pulmonary edema [50]. Pulmonary hypertension in late phase is due to platelet activating factor and leukotrienes [52, 53].

II. **Meconium aspiration model**: Meconium aspiration is one of most common causes of PPHN [49]. Meconium aspiration causes hypoxemia, respiratory acidosis and modest pulmonary hypertension by interfering with lung surfactant pools and mechanically obstructing the airways [54, 55]. This model has been developed in several animal species such as piglets [54], lambs [56], guinea pigs [57], and rabbits [58] by using a variety of concentration of meconium suspension administered intratracheally, causing acute pulmonary vasospasm.

Chronic models of PPHN

I. **Fetal lamb duct ligation model:** Surgical ligation of ductus arteriosus in late gestation fetal lambs temporary enhances pulmonary blood flow and causes substantial elevation in PVR [59]. Lambs delivered with ligated ductus develop pulmonary hypertension and shows many similarities with idiopathic PPHN, such as severe oxygen insensitive hypoxemia and the extension of smooth muscle into non-muscular distal arteries [60, 61].

II. **Chronic nitric oxide synthase inhibition:** In utero infusion of nitric oxide synthase inhibitor N omega-nitro-L-arginine in fetal lambs for ten days produces pulmonary hypertension after birth with elevated pulmonary artery pressure and resistance [49, 62]. Pulmonary arteries of these lambs are anatomically normal but do not show a relaxation response to acetylcholine. L-arginine administration to these lambs decreases pulmonary artery pressures to normal values [62].

III. **Chronic hypoxia:** Newborn piglets, when kept hypoxic for 72 hours, develop pulmonary hypertension as hypoxia interferes with a postnatal adaptation of pulmonary vasculature. Smooth muscle cells in hypoxic piglets maintain their fetal shape and have more myofilaments. Pulmonary arteries have a thick walled structure with incomplete postnatal dilation compared to control piglets [63, 64].

1.1.8 Treatment of PPHN

Optimal lung recruitment and selective pulmonary vasodilation to normalize ventilation-perfusion ratio are major aims of PPHN therapy [8].

I. Inhaled nitric oxide: Nitric oxide (NO) is a famous smooth muscle relaxant [65] and was first shown to improve oxygenation in severe PPHN patients in the year 1992 [66]. NO is usually administered with mechanical ventilation or positive airway pressure to maximize NO delivery to airways and thus get more vasodilation while maintaining best ventilation:perfusion matching [67]. Infants with extra pulmonary right-to-left shunting and without parenchymal lung disease respond best to NO therapy [68]. NO triggers rapid and selective pulmonary vasodilation through diffusion across alveoli to smooth muscle cells of pulmonary arteries to activate soluble guanylyl cyclase enzyme - which upon activation, forms cyclic guanosine monophosphate (GMP) from guanosine-5'-triphosphate (GTP) [69, 70]. Cyclic GMP activates protein kinase G (PKG), which initiates smooth muscle relaxation by acting on a variety of targets [71]. Results from large placebo-controlled trials suggest that use of NO has significantly reduced the need for extracorporeal membranous oxygenation (ECMO) support in infants with PPHN. However, NO use did not reduce mortality and length of hospitalization.

Up to 40 per cent of infants were not able to sustain NO-induced vasodilation [68]. NO is most effective in decreasing pulmonary to systemic artery pressure when given at a dose of 20 ppm [72]. Once oxygenation is improved NO is weaned slowly to prevent rebound pulmonary hypertension [73] due to inhibitory effect of NO on endothelial nitric oxide synthase activity [74].

II. Sildenafil: Sildenafil is a competitive inhibitor of phosphodiesterase (PDE) 5 enzyme, which hydrolyzes cyclic guanosine monophosphate (cGMP) and thus elevates the cellular pool of cGMP and causes smooth muscle relaxation [75, 76]. Sildenafil was initially used to help weaning NO from infants who underwent surgery for congenital heart disease [77]. Later oral and intravenous sildenafil was shown to improve oxygenation and survival in infants with PPHN [78, 79]. Sildenafil is a promising choice for therapeutic management of pulmonary hypertension secondary to CDH and BPD as it has low toxicity and can be given orally [68].

III. Prostacyclin: Prostacyclin is an endogenous vasodilator prostanoid, which stimulates prostacyclin receptors on smooth muscle cells, and mediates vasodilation via the cyclic adenosine 3'-5'monophosphate (cAMP)-protein kinase A (PKA) pathway [80]. Inhaled prostacyclin can improve oxygenation and decreases the need for ECMO in critically ill infants with PPHN, who are not responsive to NO treatment [68, 81]. However, these studies are limited to small populations [82, 83] and at present optimal dosing and formulation of inhaled prostacyclin needs improvement, as the current formulation is an airway irritant and with a short half life [68]. To overcome these issues other synthetic

prostacyclin analogs such as inhaled iloprost and treprostinil have been introduced, which have longer half-lives and can be made in less irritable formulation [67]. A retrospective study reviewed 19 PPHN cases and suggested that inhaled iloprost could be used as an alternative PPHN therapy without observing systemic hypotension [84].

IV. Milrinone: Cellular cAMP levels are controlled in part by PDE 3 enzyme, which hydrolyzes cAMP to terminate its vasodilatory effects. Milrinone maintains and promotes vascular smooth muscle relaxation by increasing cAMP availability by inhibiting PDE 3 [85, 86]. Use of milrinone has increased in neonatology over the last ten years [87]. Intravenous milrinone improved oxygenation and heart rate in infants with NO refractory severe PPHN; no systemic hypotension was observed [88]. However a recent retrospective data analysis done on a larger infant population suggest that milrinone use is associated with hypotension and thrombocytopenia as major adverse events [87].

V. Bosentan: Endothelin (ET) is an endothelium-derived peptide and triggers profound pulmonary vasoconstriction via acting on smooth muscle localized ET_A receptors [89, 90]. Bosentan is a dual ET receptor antagonist [91] and was initially used successfully in 2 infants with PPHN as a result of transposition of great arteries [92]. Later bosentan was used in one more infant with PPHN who showed improvement in oxygenation [93]. A randomised controlled trial is currently studying the efficacy of bosentan in infants with severe PPHN [67].

1.2 G-protein coupled receptor (GPCR) signaling in PPHN

1.2.1 Prostanoid receptors

The cellular membrane is composed of proteins and lipids, including phospholipids. Of these phosphatidylethanolamine and phosphatidylcholine are major phospholipids in the cellular membrane [94] . Calcium release due to chemical or physical stimuli triggers translocation of α-isozyme of cytosolic phospholipase A_2 to various cellular locations such as the perinuclear region [95, 96] and golgi membrane [97]. Phospholipase A_2 hydrolyzes the sn-2 ester bond of membrane phospholipids (mainly phosphatidylcholine) and causes the release of arachidonic acid (AA) [98, 99]. AA once released undergoes a cyclizing reaction to give prostaglandin G_2 (PGG_2), followed by reduction of the 15-hydroperoxy group of PGG_2 yielding immediate prostaglandin precursor prostaglandin H_2 (PGH_2) through the action of endoplasmic reticulum lumen-associated dimeric cyclooxygenase (COX) enzymes COX 1 and COX 2 [100-102]. PGH_2 is finally converted to prostaglandins such as prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane (TxA_2) by their respective synthase enzymes. These prostanoids act as ligands for several G-protein coupled receptors such as DP, EP1, EP2, EP3 and EP4, FP, IP, and TP [103]. Of these IP, EP2, EP4 and DP1 are involved in vasorelaxation and EP1, EP3, FP and TP are contractile receptors [102] **(Figure 1.1).** Prostaglandins are short-lived chemicals and act in a paracrine and autocrine fashion to display their biological effects. Prostanoids are cleared from circulation by energy-dependent uptake followed by cytoplasmic oxidation by prostaglandin dehydrogenase and reductase enzymes in lungs and liver [104, 105] an exception is prostacyclin (PGI_2) which is metabolized in the liver but escapes pulmonary inactivation [106]. Thromboxane was initially characterized as rabbit aorta contracting substance [107] and later found to be synthesized from short-lived PGH_2 by the enzyme thromboxane synthase [108]. Thromboxane is

an evanescent chemical with a half-life of 30 seconds [109]. Thromboxane spontaneously degrades into thromboxane B2 while triggering its various biological effects such as vasoconstriction [109], platelet aggregation [110], and cell proliferation [111].

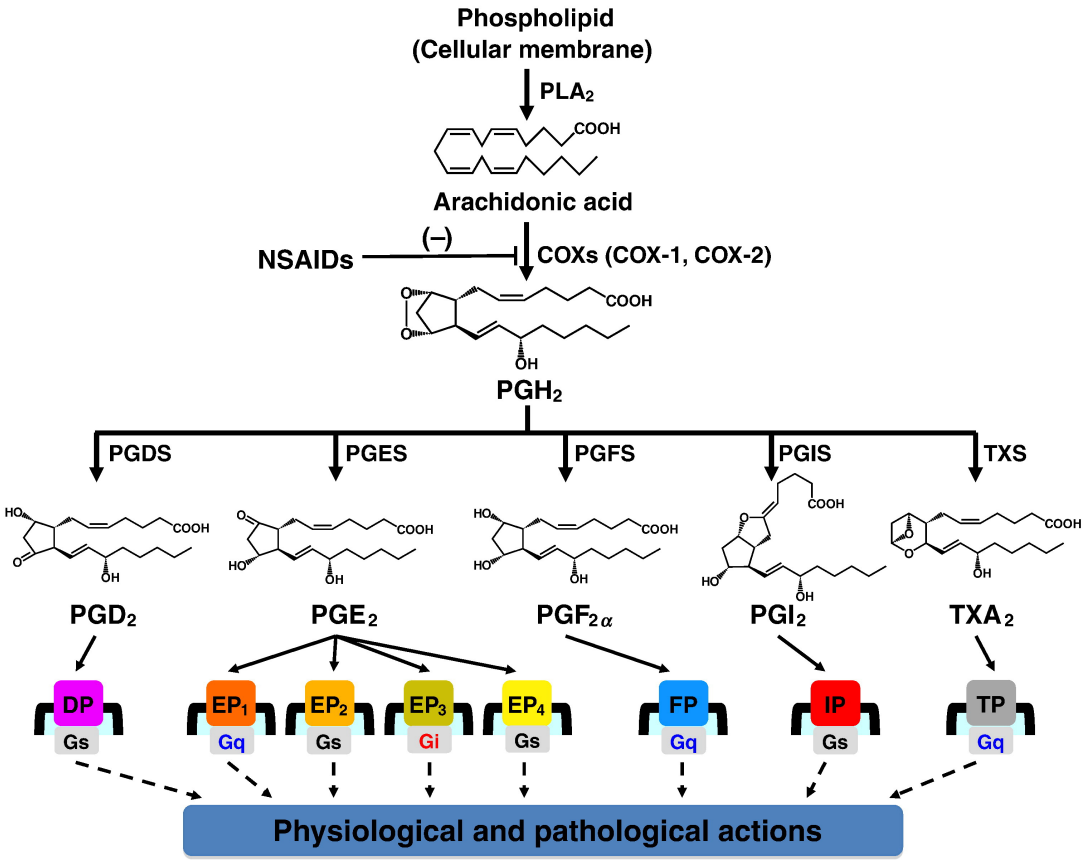

Figure 1.1 Biosynthetic pathway and receptors of prostanoids.

COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; PGDS, PGD synthase; PGES, PGE synthase; PGFS, PGF synthase; PGIS, PGI synthase; PLA2, phospholipase A2; TX, thromboxane; TXS, TX synthase.

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1.2.2 Thromboxane prostanoid (TP) receptors

Thromboxane receptors are members of the super family of G-protein coupled receptors that are membrane proteins with seven trans membrane domains connected by three extracellular and three intracellular loops [103]. TP is present in almost all organ systems in the body. At present it is known to exist in 2 isoforms in humans, namely TPα and TPβ, which are splice variants of a single gene [112]. However, until now, only TPα has been reported in other animal species. TPα was first isolated from human placenta [113] and TPβ from endothelial cells [112]. TPα and TPβ proteins differ in their amino acid composition, organ system distribution, G-protein coupling, post-translational modifications and physiological functions [114]. TPα protein is composed of 343 amino acids whereas TPβ has 407 amino acids with a longer C-terminus compared to TPα [114]. Expression of TPα generally predominates TPβ, and TPα is the major isoform present in vascular smooth muscle cells and platelets [115]. The primary endogenous ligand for TP receptors is thromboxane. However PGG2, PGH2, PGD_2, isoprostane 2α-III are also known to stimulate TP receptors [116, 117]. Due to the short half-life of thromboxane, several synthetic ligands of TP receptor have been developed, of which U46619 is extensively used to study TP receptor-mediated cellular effects [118]. SQ29548 is used as a TP antagonist [119], but a recent report studying constitutive active mutant of TP receptor A160T suggested that SQ29548 acts as a TP receptor inverse agonist [120]. Ramatroban and seratrodast are other TP antagonists [121]. Studies with point mutations in trans membrane (TM) regions of TP receptor suggest that all TM regions of TP receptor except TM2 participate in ligand binding interactions [122]. However a study using SQB, a biotinylated ligand of TP receptor, suggests ligand binding occurs near the extracellular membrane surface [123]. Later studies revealed the Cys183-Asp193 region on extracellular loop 2 (ECL2) is involved in ligand binding as well as inhibition of platelet

aggregation [124]. Nuclear magnetic resonance prediction and mutagenesis of residues on ECL2 revealed Val^{176}, Leu^{185}, Thr^{186} and Leu^{187} are the residues most important for ligand binding [125]. Intracellular loop 2 (ICL2) and ICL3 of TP receptor play a predominant role in G-protein coupling and signalling [126]. Arg^{60} in ICL1, Arg^{130} and Phe^{138} in the ICL2, and Cys^{223} in the ICL3 are critical residues forming the putative G-protein binding pocket [127]. A recent study using site directed mutagenesis, found that Thr^{135}, Arg^{136} and Arg^{147} of ICL2 are essential for TP coupling with Gαq [126].

I. Signal transduction through TP receptors

Thromboxane is one of the many prostaglandins released from endothelial and smooth muscle cells as a product of arachidonate cascade [128]. Cellular actions of thromboxane following stimulation of TP receptors are mediated through their coupling with various G-proteins such as Gαq, Gαs, Gαi, $G\alpha_{11,}$ $G\alpha_{12,}$ $G\alpha_{13}$ [129-132]. Besides this TP also interacts with $G\alpha_{15}$ and $G\alpha_{16}$ G-proteins [133]. TP receptor-mediated vascular smooth muscle contraction and mitogenic effects involve TP receptor coupling with Gq, $G\alpha_{11,}$ $G\alpha_{12}$ and $G\alpha_{13}$ proteins. TP receptor stimulation follows the canonical pathway of inositol 1,4,5-triphosphate (IP_3) generation [134] causing release of intracellular calcium from endoplasmic reticulum (ER) initiating myosin light chain phosphorylation and actin-myosin cross bridge cycle [134, 135]; and Rho A activation causing Rho kinase-mediated calcium sensitization of contractile apparatus [135]. The role of extracellular and intracellular calcium in TP agonist-mediated vasoconstriction varies with vascular preparation as well as with animal species under study. For example in rat aortic smooth muscle cells (SMCs) and aortic rings, blocking extracellular calcium does not affect intracellular calcium elevation and force generation following U46619 stimulation [134] However in another study in rat aorta, U46619 stimulated contraction was partially dependent on extracellular

calcium entry via calcium channels including both L-type and non L-type [136]. In cat coronary arteries thromboxane mediated vasoconstriction requires extracellular calcium [137]. In bovine and rat pulmonary arteries U46619-mediated vasoconstriction is sensitive to Rho kinase inhibition, chloride channel blockade, and store operated calcium channel blockade; and remains unaffected by voltage operated calcium channel blockade [138, 139]. In rat pulmonary arteries U46619-mediated vasoconstriction involves PKCς mediated inhibition of Kv channels and requires calcium entry through L-type calcium channels [140].

Besides this, TP receptor has also been shown to promote vasoconstriction independent of G-proteins in a non-canonical way. TP receptor co-localizes and interacts with the BK channel α and regulatory β subunits [141, 142]. In human coronary arteries and in rat aorta, TP receptor, and BK channel shows proximity and U46619 stimulation follows TP receptor to inhibit BK channel independently of G-protein activation favoring vasoconstriction whereas regulatory β subunit of BK channel inhibits TP-mediated inhibition of BKα subunits and decreases vasoconstriction [141, 142]. Besides vasoconstriction, TP receptor is also implicated in proliferation and hypertrophy of vascular smooth muscle cells. TP receptor stimulation with U46619 increases proliferation of rat aortic smooth muscle cells by mitogen-activated protein kinase (MAPK) activation and expression of immediate growth response genes [111, 143]. This may be preparation-dependent however, as other studies on rat [144] and bovine aortic [145] and bovine coronary [146] artery smooth muscle cells found that TP agonists have minute mitogenic effects. TP agonist U46619 shows synergistic proliferative effects with thrombin [147], serotonin [148] and growth factors such as platelet-derived growth factor (PDGF) [146] to stimulate vascular smooth muscle cell proliferation. TP receptor is also involved in vascular smooth muscle hypertrophy through various mechanisms including up-regulation of basic fibroblast

growth factor [149] and PKC-mediated elevation of transforming growth factor β (TGFβ) [150]. In the bovine pulmonary vasculature, U46619-mediated proliferative effects involve activation of P^{38}-MAPK phosphorylation and nuclear factor-κB (NF-κB) activation [151].

II. Role of TP receptor in PPHN

Thromboxane levels remain low during pregnancy but are elevated just before delivery to promote closure of umbilical vessels and ductus arteriosus [152]. Thromboxane mediates the early peak in pulmonary artery pressure and resistance in juvenile pigs treated with human meconium [153], in piglets treated with group B streptococci [154] as well as in chronic hypoxic piglets [155]. Treatment of piglets with TP receptor antagonist terbogrel lessen the chronic hypoxia-induced early rise in pulmonary pressure and improves acetylcholine-induced pulmonary vasodilation [156]. Thromboxane contributes to acetylcholine-induced pulmonary vasoconstriction in chronic hypoxic piglets [157].

TP receptors antagonism attenuates the hyperoxia-induced endothelin-1 production and subsequent pulmonary hypertension in newborn rats [158]. Neonatal swine pulmonary artery myocytes have a higher resting calcium level and more polymerized actin than aortic myocytes, and following three days of hypoxic exposure show a pronounced calcium mobilization and actin polymerization response to TP receptor stimulation compared to normoxic myocytes [159, 160]. Chronic hypoxia decreases cell surface density of TP receptor while increasing receptor affinity in piglet pulmonary artery myocytes [161] and pulmonary artery rings from hypoxic piglets show increased force generation to TP receptor stimulation [162]. TP receptor agonist U46619 decreases proliferation of neonatal swine pulmonary artery myocytes and promotes them to enter into more contractile phenotype [163]. Duct ligated pulmonary hypertensive lambs have higher

levels of thromboxane metabolite TxB2 in lung tissues [164]. Further, as reported recently in these lambs, thromboxane synthase expression and activity increases in pulmonary artery endothelial cells and this is associated with decreased angiogenesis [165].

Conclusions on the role of thromboxane drawn from animal and in vitro PPHN models are also borne out in human studies. Newborn infants with hypoxemic respiratory failure have lower PGE_2/ TxB_2 ratio compared to normal infants indicating increased levels of thromboxane generation [166]. In newborn infants with pulmonary hypertension associated with meconium aspiration, plasma TxB_2 levels as well as pulmonary artery pressure decrease following ECMO; successful ECMO treatment or recovery from PPHN were correlated with a reduction in plasma TxB_2 levels [167]. These data, in addition to the many detailed mechanistic studies above, confirm that thromboxane is a key etiologic factor in the development of PPHN, whether induced by hypoxia, inflammation or pulmonary vascular overflow.

1.2.3 Isoprostanes

Free radical catalyzed cyclooxygenase-independent or dependent lipid peroxidation of esterified or free polyunsaturated fatty acid (primarily arachidonic acid) generates isoprostanes *in vitro* or *in vivo* [168]. In a healthy individual, isoprostane levels are very low in the plasma and urine [169]. However, under conditions of increased oxidative stress such as hypertension, atherosclerosis, cancer and aging, an increase in free radical species inside the cells leads to a several-fold increase in isoprostane levels in plasma and urine of affected individuals [170]. Preeclampsia, intrauterine growth retardation, pulmonary hypertension and BPD are also associated with increased isoprostane levels in biological fluids [171]. In the cardiovascular system, isoprostanes are implicated in vasoconstriction, platelet aggregation, angiogenesis and

adhesion of inflammatory cells to vascular endothelium[169]. Cyclopentane ring isoprostanes are described into several classes such as D2-isoprostane, E2-isoprostane, F2-isoprostane, A2-isoprostane, and J2-isoprostane [172]. Plasma F2-isoprostanes are higher in preterm babies than term babies, which have higher levels of F2-isoprostanes than adults; this suggests that there is an inverse correlation between plasma F2-isoprostanes and gestational age [173].

1.2.4 Effects of isoprostanes on newborn pulmonary circuit

Isoprostanes can constrict or relax pulmonary as well as systemic vascular smooth muscle, depending on the type of the isoprostane and species and tissue under study [168, 174]. F and E-ring isoprostanes constrict newborn and 2-week-old piglet pulmonary arteries and veins (less potently than the thromboxane mimetic U46619); this constriction is abolished in the presence of TP receptor antagonism by SQ29548, and appears to be mediated through tyrosine kinase and Rho kinases [175]. 8-iso-PGF2α constricts adult pulmonary arteries more potently than newborn rat pulmonary arteries, through stimulation of TP receptors [176]. 8iso-PGE2 causes an age-dependent increase in relaxation of U46619 precontracted piglet pulmonary arteries, as well as SQ29548 treated pulmonary arteries, via EP receptors and nitric oxide [177]. In term mice, 8-isoPGF2α and 8-isoPGE2α constrict the ductus arteriosus via stimulation of TP receptors; however in the presence of TP antagonism, EP4 receptor-mediated dilation occurs. In contrast, the ductus arteriosus of preterm mice shows dilation following stimulation with 8-iso-PGE2α irrespective of TP antagonism [178]. Pulmonary artery myocytes of hypoxic newborn swine have reduced superoxide dismutase activity, leading to the formation of 8-isoprostane via peroxynitrite [179]. F2-isoprostanes are also increased in 3-day hypoxic piglet resistant pulmonary arteries, however, this increase returns to normal levels following ten days of hypoxic

exposure [180]. However in newborn rat exposed to 60% oxygen for 14 days, 8-iso-PGF2α levels increases, associated with increased contractile responses of pulmonary arteries [181]. In duct-ligated PPHN lambs, treatment with recombinant human superoxide dismutase and inhaled NO results in improved oxygenation and thereby, reduced lung isoprostanes levels [182].

In summary, Isoprostanes are involved in both hypoxic and hyperoxic pulmonary hypertension models. Isoprostanes have low potency compared to U46619, a standard TP agonist. The functional effects of isoprostanes are variable, binding to different receptors and acting through many alternative pathways. Isoprostanes dilate the preterm ductus; however contraction occurs in term ductus. Isoprostanes promote pulmonary arterial contractile responses mainly through TP receptors. Certain isoprostanes may also show relaxation responses in precontracted pulmonary arteries via Rho kinase and nitric oxide pathways.

1.2.5 Prostacyclin receptor (IP)

In 1976, Vane and coworkers discovered that the porcine aorta releases a substance prostaglandin X (PGX) that is capable of relaxing mesenteric and coeliac arteries and inhibiting platelet aggregation [183]. The chemical structure of PGX was studied later, and the new name of prostacyclin was given to this compound [184]. Cell membrane derived arachidonic acid is catalyzed to prostacyclin in a stepwise manner by cyclooxygenase and prostacyclin synthase enzyme [185]. Prostacyclin synthase is present on cellular as well as nuclear membranes of vascular and nonvascular smooth muscle cells [186]. Nuclear signaling of prostacyclin involves activation of peroxisome proliferator-activated receptors [187].

The half-life of prostacyclin is about three minutes [188], due to this reason more stable analogs have been synthesized for prolonged stimulation of IP receptors. These include but not limited to epoprostenol, ciprostene, UT-15, iloprost, cicaprost, beraprost [189] and a non-prostanoid agonist selexipeg, which gives an active metabolite MRE-269 – an extremely selective IP agonist [190].

I. Vascular smooth muscle relaxation through IP-AC-cAMP pathway

Prostacyclin is an endogenous ligand for the G-protein coupled prostacyclin receptor, which is termed as IP [191, 192]. IP is composed of 386 amino acids giving a molecular weight of 37-41 kDa, and has a short N-terminus and an extended C-terminal tail [192]. In the vascular system IP is present on plasma membranes of endothelial and vascular smooth muscle cells. There is only one cloned subtype of IP receptor, however; binding and functional studies have identified another type of IP receptor (IP2) in airway epithelial cell line [193] and central nervous system [194]. The IP receptor mainly couples with Gαs to mediate its cellular effects; however, studies

from mammalian cell lines using synthetic IP agonists suggest that IP also interacts with Gαi [195] and Gαq [196].

In a healthy person, cyclooxygenase-2 enzyme is the primary source of systemic prostacyclin [197]. Vascular endothelial cells are the main producers of PGI2 in the body [198]. Endothelial cell-derived prostacyclin diffusion through myoendothelial junctions activates vascular smooth muscle localized prostacyclin receptors to initiate vasorelaxation, whereas diffusion to the vessel luminal side inhibits platelet aggregation and thrombus formation in blood [199]. Stimulation of prostacyclin receptors by PGI2 and its analogs activates the adenylyl cyclase enzyme via stimulatory G-protein Gαs. There are ten isoforms of adenylyl cyclase (AC) enzyme (AC1 to AC10) of which, nine (AC1-AC9) are transmembrane proteins, whereas AC10 is cytosolic [200]. Membrane adenylyl cyclase enzymes are stimulated or inhibited by various second messengers and G-proteins such as Ca^{2+}-calmodulin stimulates AC1, AC3, and AC8 [200, 201]. Gβγ proteins activate AC2, AC4, and AC7 whereas Ca^{2+}, Gαi, and Gβγ proteins inhibit AC5 and AC6 [202]. Gαs and forskolin stimulate all isoforms of membrane adenylyl cyclases except AC9, which is resistant to activation by forskolin [200]. Adenylyl cyclase utilizes cellular adenosine triphosphate (ATP) to generate second messenger cAMP. cAMP alone or by activation of the enzyme protein kinase A increases the open probability of various K^+ channels such as BKca channel in guinea pig aorta [203], K_{ATP} and Kca channels in isolated rat lung [204] and TWIK-related acid-sensitive K^+ channel 1 (TASK-1) in human pulmonary artery smooth muscle cells [205]. Activation of K^+ channels leads to hyperpolarization of the cell, which initiates smooth muscle relaxation by decreasing calcium entry through L-type calcium channels [80].

A recent study on isolated human pulmonary artery suggested that treprostinil could be the best choice for achieving pulmonary arterial relaxation in pulmonary hypertension (PH) patients with higher plasma levels of TxA2 metabolites; whereas PH patients with higher ET-1 levels, both iloprost and treprostinil will give efficient pulmonary artery relaxation [206]. In precontracted rat aorta, lower doses of PGI2 induce a relaxation response but at higher concentrations reversal of relaxation occurs; this reversal of relaxation is abolished in the presence of SQ29548 (a TP receptor antagonist), suggesting that at higher concentration PGI2 stimulates TP receptors in rat aorta [207]. Higher concentrations of PGI2 also constricts cat and human [208] and dog cerebral arteries [209].

II. Inhibition of proliferation by IP receptor

Besides causing pulmonary vasodilation, prostacyclin signaling also inhibits proliferation of pulmonary artery smooth muscle cells. IP knock-out mice at high altitude develop more severe pulmonary hypertension with a more thickened pulmonary arterial media compared to wild-type mice [210]. Prostacyclin analogs UT-15, iloprost, cicaprost and beraprost inhibit the serum-induced proliferation of human pulmonary artery smooth muscle cells, by promoting cAMP generation [211]. Cicaprost- and iloprost-generated cAMP inhibit platelet-derived growth factor (PDGF) -BB-induced DNA synthesis and show antiproliferative effects more potently in distal than proximal human pulmonary artery smooth muscle cells (PASMCs) [212]. Further, cicaprost arrests the fetal calf serum-stimulated proliferation of primary human PASMCs [213]. Cicaprost also reduces the cytokine-induced ET-1 release in human PASMCs [213]. Another PGI2 analog, beraprost sodium, and the cAMP analog 8-br-cAMP both inhibit the proliferation of hypoxic and normoxic serum-fed human PASMCs, via a cAMP-mediated fall in p27kip1mRNA degradation [214]. In human PASMCs, iloprost and treprostinil increase expression of the bone

morphogenetic protein 4 (BMP4) target gene id1, via cAMP-PKA pathway acting on a cAMP response element binding protein site on id1 gene promoter [215]. Further, in human PASMCs from pulmonary arterial hypertension (PAH) patients harboring a mutation on BMP receptor 2 gene, iloprost treatment increased BMP4-stimulated smad1/5 signaling and id protein expression and thus decreases the proliferative potential of these cells [215]. Hypoxia increases proliferation of human PASMCs by increasing expression of arginase-2 [216]. In this context, cAMP elevating agents such as 8-br-cAMP, forskolin and PDE 3 inhibition by cilostamide all inhibit hypoxia-induced proliferation of human PASMCs by the action of exchange protein directly activated by cAMP (EPAC)-mediated inhibition of arginase-2 in hypoxic PASMCs [217]. However, antiproliferative effects of IP agonists also include stimulation of nuclear peroxisome proliferator-activated receptor γ (PPARγ) receptors in idiopathic pulmonary hypertension patients [218].

Taken together, it appears that IP receptor signaling is essential to limit the proliferation of pulmonary artery smooth muscle cells at physiological levels, and impaired IP signaling contributes to pulmonary artery remodeling in pulmonary hypertension. IP receptor agonists are capable of reducing proliferation of PASMCs induced by various triggers such as serum, hypoxia, cytokine stimulation, and genetic loss of BMP receptor gene; in the large part, the mechanism of inhibition of proliferation appear to be by raising cellular cAMP.

III. Prostacyclin signaling in PPHN

Prostacyclin is generated by air-filled distension of lungs with the initiation of respiration at birth plays a role in pulmonary vasodilation [219] and also promotes surfactant secretion by type II alveolar epithelial cells [220]. Pulmonary artery infusion of PGI2 induces potent pulmonary

vasodilation in fetal goat and sheep [221]. In ovine pulmonary endothelial cells and smooth muscle cells, PGI2 synthesis increases from late fetal to newborn life, and this increase is mediated by concomitant increased expression of cyclooxygenase-1 protein from fetal to newborn life [222]. Increased pulmonary COX-1 protein and thus increased PGI2 in late gestation ovine fetus is due to placenta-derived estrogen hormone [223]. The postnatal decrease in glucocorticoids further increases COX-1 protein expression and PGI2 synthesis [224]. In ovine intrapulmonary arteries, oxygen triggers the PGI2 production in the fetal life however decreased oxygenation stimulates PGI2 production in postnatal life [225]. Lungs of duct ligated fetal lambs [164], as well as pulmonary arteries of chronic hypoxic PPHN piglets [155] have decreased levels of prostacyclin metabolite 6-keto-prostaglandin F1α (6-keto-PGF 1α) than thromboxane metabolite TxB2. Newborn calves, which are exposed to chronic hypobaric hypoxia, develop severe pulmonary hypertension and show reduced PGI2 and PGE2 production in proximal and distal pulmonary arteries [226].

Chronic hypoxia does not alter expression of the prostacyclin synthase enzyme but increases the expression of thromboxane synthase in resistance pulmonary arteries of piglets [227]. In a fetal lamb duct ligation model of PPHN, intratracheal inhaled iloprost and intravenous milrinone increased pulmonary blood flow by decreasing elevated pulmonary artery pressure and resistance, suggesting the impairment of IP receptor signalling in PPHN [228]. A recent study indicates that duct ligation in fetal lambs decreases prostacyclin synthase enzyme expression and activity, and increases thromboxane synthase enzyme expression and activity in pulmonary artery endothelial cells, leading to impaired angiogenesis in PPHN lambs compared to controls [165]. Thus, studies from various PPHN animal models suggest that prostacyclin plays an

important role in perinatal transition by promoting vasodilation and surfactant secretion and keeping a check on thromboxane-mediated vasoconstriction.

1.2.6 Prostanoid E (EP) receptors

Microsomal, cytosolic and membrane-associated prostaglandin E synthase enzymes generate prostaglandin E2 in the body [229]. In vascular smooth muscle cells, microsomal PGE synthase is the major enzyme responsible for PGE2 production [230]. Prostaglandin E2 is the most abundant prostanoid in the animal kingdom and involved in a variety of functions such as regulation of blood pressure, immune response, protection of gastrointestinal mucosa, and pain sensation via stimulation of several EP receptors [231-233]. EP receptors are of four types namely EP1, EP2, EP3 and EP4; of these, EP3 has multiple isoforms due to alternative splicing in mice, rat, bovine and human [234]. Candidate G-proteins for the EP1 receptor are not clear, and reported data may depend on particular cell type studied [103]. EP1 receptor couples with Gαq and mobilizes intracellular calcium in HEK293 cells, and up-regulates hypoxia-inducible factor 1α (HIF1-α) in a hepatocarcinoma cell line via coupling with Gαi/o proteins [235]. EP2 and EP4 receptors mainly stimulate Gαs to generate cAMP whereas the primary G-protein for the EP3 receptor is Gαi [236]. EP2 and EP4 receptors can also function independently of G-proteins by forming a complex with β-arrestins that can recruit other proteins such as Src complex, PKA and epidermal growth factor receptor [237, 238]. Tissue distribution studies for EP receptor mRNAs in mice suggest that EP1 expression is predominant in kidney followed by lungs [239] whereas EP2 expression is most abundant in the uterus [240]. EP3 and EP4 have a wide organ distribution in mice [241].

I. Effect of PGE2 on contractility of systemic smooth muscle

Depending on the particular EP receptor involved, the effect of PGE2 on the vascular bed could lead to local vasodilation or vasoconstriction. Generally speaking, EP1 and EP3 act as vasoconstrictors opposed by the dilator action of EP2 and occasionally EP4; the balance of these opposing signals maintains basal tone in response to PGE2 stimulation. PGE2 dilates precontracted rabbit isolated ear artery and saphenous vein [242]. Endothelium-dependent contractile effects of PGE2 in the spontaneously hypertensive rat aorta involve EP1 receptors [243]. In human aortic smooth muscle cells, EP2 receptors antagonize histamine-evoked calcium signals [244]. Intravenous infusion of PGE2 into mice produces rapid but transient hypotension, which is mediated primarily by EP2 receptors. EP2 knock-out mice show an EP3 mediated hypertensive effect following PGE2 infusion [245]. PGE2 induced constriction of porcine large cerebral arteries involves EP1 and EP3 receptors [246]. In guinea pig aorta, sulprostone induced constriction involves EP3 receptors [247]. In humans, PGE2 constricts the intercostal arteries via EP3 receptors [248].PGE2 also constricts the afferent renal arteriole via EP3-Gαi signalling, and relaxes the same via EP4-Gαs signalling [249]. PGE2 constricts the internal mammary artery of humans via EP3, and relaxes the human saphenous vein and middle cerebral arteries via EP4 receptors [250, 251]. There are also some mixed effects due to cross-talk or mixed signalling of ligands via several receptors. EP1 receptor constricts, and EP2 relaxes guinea pig trachea; [252]. In rat lungs, local epithelial injury induces global airway contraction involving adenosine A1 and EP3 receptors [253]. In rat mesenteric artery PGE2-induced vasoconstriction is a result of EP3, FP and TP receptor stimulation by PGE2 via PKCδ, PKCε and Rho kinase pathways [254]. In epithelium intact airways, glucagon-induced airway smooth muscle relaxation is mediated by

nitric oxide and PGE2 [255]. EP1 receptor constricts, and EP2 relaxes guinea pig trachea and thus maintain balanced basal tone [252].

II. Effect of PGE2 on contractility of pulmonary vascular smooth muscle

PGE2 does not affect the contractility of isolated rat pulmonary arteries [256]. In isolated human pulmonary arteries, PGE2 and sulprostone induce contraction via EP3 receptors [257, 258]. Iloprost-mediated cAMP generation in pulmonary artery tissue from PAH patients and in MCT-treated rats involves mainly EP4 receptors, as in these tissues IP receptor expression is reduced and thus cannot contribute to cAMP generation [259]. A recent study suggests that contractile EP3 receptors are upregulated by hypoxia in human distal pulmonary arteries and MCT-treated rodents and trigger remodeling of pulmonary arteries via Rho-TGFβ1 pathway [260]. Iloprost-mediated relaxation of human pulmonary veins remains lower than arteries due to concomitant activation of contractile EP1 receptors [261]. In human pulmonary veins, EP4 receptor is responsible for PGE2-induced relaxation [262].

III. Role of PGE2 in newborn pulmonary circulation

Placenta and ductus arteriosus derived PGE2 keeps the ductus arteriosus dilated in fetal life, which allows blood to bypass the high resistance pulmonary circulation [263, 264]. PGE2 concentration in the newborn decreases as compared to fetal life, and this together with enhanced oxygenation of blood initiate duct closure [265]. Ductus arteriosus myocytes express EP2, EP3 and EP4 receptors, of which EP4 expression is most abundant in media of fetus and newborn ductus [265, 266]. Ductal smooth muscle but not the ductal endothelial EP4 receptor plays an essential role in postnatal duct closure [267]. EP4 receptor-deficient mice have a patent ductus arteriosus (PDA) and die of heart failure as newborns [268]. PGE2 acting on ductal EP4

receptors promotes intimal thickening via adenylyl cyclase 6-cAMP [269] and Epac1 pathway [270] whereas the same stimulus inhibits ductal elastogenesis through EP4 phospholipase Cγ stimulated lysyl oxidase degradation [271].

Pulmonary artery infusion of prostaglandin E1 and E2 to newborn goats decreases pulmonary pressure and resistance [272] and a PGE1 infusion also prevents the hypoxic pressor response in newborn goats [273]. Infants who get pulmonary hypertension secondary to neonatal hypoxemic respiratory failure have lower plasma PGE2/Thromboxane B2 ratio compared to normal infants [166], suggesting a role for EP receptors in regulating pulmonary arterial resistance.

IV. Prostaglandin E1

Besides metabolizing arachidonic acid, cyclooxygenase enzymes also utilize a polyunsaturated fatty acid as a substrate called dihomo-γ-linoleic acid (DGLA) to produce series-1 prostaglandins such as prostaglandin E1 (PGE1). Endogenous generation of PGE1 is limited as the majority of the cellular unsaturated fatty acid pool is composed of arachidonic acid, which the cyclooxygenase-1 enzyme catalyzes preferentially over DGLA (COX-2 utilizes both substrates equally). PGE1 has a half-life of 30 seconds in humans [274] PGE1 stimulation causes cAMP generation in the cell [275], resulting in vasodilatory [276], inotropic [277] and antiproliferative [275] platelet anti-aggregatory [278], and angiogenesis stimulatory properties [279]. PGE1 stimulates IP and EP3 receptors on platelets [280]. Clinical uses of PGE1 include emergent intervention to preserve ductal patency in ductus dependent congenital heart disease [281], alleviating right ventricular afterload in neonatal hypoxemic respiratory failure by permissive right to left ductal shunting [282] and in the treatment of residual pulmonary hypertension

following corrective heart surgery [283]. A recent report suggested that the use of PGE1 to open the ductus arteriosus is associated with an up to 75 percent reduction in length of hospital stay in PPHN patients compared with patients with duct dependent congenital heart disease treated with the same agent, suggesting the possible utility of PGE1 in PPHN treatment [284].

1.2.7 Prostaglandin D2 receptors

Isomeric conversion of prostaglandin H2 to prostaglandin D2 is catalyzed by the lipocalin-type or the hematopoietic type of the enzyme prostaglandin D synthase [285]. PGD2 is the principal prostanoid present in the brain, where PGD2 plays a role in sleep promotion [286] and pain perception [287]. Mast cells are the main producers of PGD2 in the body [288], and PGD2 also plays a significant role in allergic inflammation in asthma [289]. Biological functions of PGD2 are mediated through DP1, DP2 and TP receptors [290]. Most of the cellular effects of PGD2 are signaled through the DP1 receptor, which couples with Gαs and increases cellular cAMP production. DP1 receptor is present on vascular smooth muscle cells and platelets, and PGD2 stimulation of DP1 receptors leads to vasodilation and inhibition of platelet aggregation[102]. DP2 is also known as chemoattractant receptor homologous molecule expressed on T-helper type 2 cells (CRTH2), a member of the family of chemokine receptor present on Th2 lymphocytes, eosinophils, and basophils [291]. PGD2 is metabolized via sequential loss of two water molecules to give PGJ2 and 15d-PGJ2 [292]. 15d-PGJ2 has potent anti-inflammatory properties and can activate DP and PPARγ receptors [292, 293].

I. Effects of PGD2 on pulmonary circuit

Pulmonary artery infusion of PGD2 dilates the fetal goat pulmonary arteries and does not affect the systemic vascular resistance [294]. In the newborn goat pulmonary circuit, a PGD2 infusion

elicits a biphasic response of dilation at lower doses and constriction at higher doses; whereas a pulmonary artery infusion of PGD2 constricts the adult goat [294], dog and cat [295] pulmonary arteries. In isolated perfused lungs of newborn pigs, PGD2 infusion causes an age-dependent increase in PVR from day 1 to day 7 of age [296]. In adult sheep, PGD2 infusion causes pulmonary vasoconstriction, which is sensitive to thromboxane receptor blockade. Furthermore, in sheep pulmonary arteries constricted due to hypoxia and treated with the TP blocker SQ29548, PGD2 infusion causes vasodilation, indicating that in sheep the vasoconstrictor effects of PGD2 are mediated solely through its interaction with the TP receptor [297]. In guinea pig peripheral lung tissues, PGD2 stimulation also causes a contractile effect in airways, pulmonary arteries and veins via stimulation of TP receptors [117]. In the same manner, TP receptors are involved in PGD2-induced contraction of rat liver fibroblasts [298]. However in human asthmatic subjects, PGD2-induced bronchoconstriction involves both TP and DP receptors [299]. It is clear that the effects of PGD2 on the pulmonary vasculature are variable by animal model. In near term newborn lambs, a PGD2 infusion attenuated induced pulmonary hypertension by reducing pulmonary artery pressure and resistance in first three days of life; but the same infusion caused pulmonary vasoconstriction in 15-day old lambs [300, 301]. PGD2 treatment did not reduce the medial hypertrophy of pulmonary arteries in chronically hypoxic newborn rats [302]. Importantly, PGD2 treatment neither lowered the pulmonary artery pressure nor improved the oxygenation in infants with persistent pulmonary hypertension [303]. PGD2 and its agonist BW245C do not relax the norepinephrine precontracted human pulmonary arteries [304]. In adult human pulmonary veins but not in arteries, relaxation to treprostinil, an IP, DP1 and EP2 receptor agonist, is abolished in the presence of a DP receptor antagonist indicating human pulmonary venous but not arterial tone may be regulated by DP receptor signaling [305, 306]. In

summary, PGD2 effects on contractility of pulmonary vasculature vary with age, species and circuit location; and in certain species and/or organs, PGD2 effects involve TP receptors.

1.2.8 Prostaglandin F2α receptors

Prostaglandin F2α (PGF2α) is the product of prostaglandin F synthase catalyzed isomeric conversion of PGH2. PGF2α acting on FP receptors plays a seminal role in the regulation of reproduction in animals and humans. PGF2α is a potent constrictor of uterine smooth muscle and also sensitizes the contractile apparatus to calcium in human myometrium and rat aorta [307-309]. PGF2α is also involved in renal physiology, cardiac hypertrophy and has a hypotensive effect on intraocular pressure. Circulating PGF2α is degraded during transit through the pulmonary circulation [310]. PGF2α is a mitogen and constrictor of smooth muscle cells [311] and induces inotropy and hypertrophy in the heart [312, 313]. PGF2α can also stimulate EP1 and EP3 receptors [314]. Stimulation of the FP receptor mainly activates Gαq protein and raises intracellular calcium [315]. In cat esophageal sphincter smooth muscle PGF2α is seen to activate Gαq and Gα13 proteins [316]. Gαq activation is followed by PKC and MAPK activation. However, the FP receptor also activates MAPK via Gαi proteins [317].

I. Effect of PGF2α on pulmonary circuit

PGF2α treatment constricts the pulmonary arteries in dogs [318, 319], lambs, pig [308] and humans [320]. PGF2α infusion causes vasoconstrictive pulmonary hypertension in dogs [321]. PGF2α constricts the pulmonary arteries from newborn swine [322]. However the contractile response of pulmonary artery rings from newborn pigs with PPHN (exposed to hypobaric hypoxia from birth to three days) to PGF2α does not change as compared to normal piglets

[323]. Proximal pulmonary arteries from hyperoxic (85% oxygen for seven days) adult and weanling rats are sensitized and hyperreactive to PGF2α stimulation [324], indicating this prostanoid may play a role in vasoconstrictor responses of the pulmonary circuit after the application of an oxidative stress.

Both thromboxane and PGF2α levels are increased in infants with pulmonary hypertension [325, 326]. But PGF2α is also elevated in infants with a patent ductus arteriosus but normal pulmonary arterial resistance [327]. Thus, while serum thromboxane has been directly and indirectly implicated in the etiology of pulmonary hypertension, there is not sufficient evidence to link PGF2α as a causative agent in this disease.

1.2.9 Endothelin receptors

Endothelin is a potent 21 amino acid vasoconstrictor peptide, initially isolated from conditioned medium of cultured endothelial cells [89]. The endothelin (ET) family consists of three members: ET-1, ET-2 and ET-3 [328]. In the vascular system ET-1 is the principal isoform secreted by endothelial cells constitutively as well as in a stimulated manner [329]. Secreted ET-1 is the product of two proteolytic steps catalyzed by an endopeptidase, which converts the ET-1 precursor peptide pre-pro-endothelin-1 to bigET-1[330]. BigET-1 is further cleaved into ET-1 by endothelin-converting enzymes [331]. ET-1 plays a significant role in the maintenance of vascular tone [332]. ET-1 has proinflammatory properties [333], and it can markedly potentiate the proliferative effects of growth factors on vascular smooth muscle cells [334]. Nitric oxide and ET-1 share an autocrine feedback loop and each counteracts the other's secretion by the endothelial cell [335]. Endothelin exerts its biological actions through two endothelin receptors, ET_A and ET_B [90, 336]. In the vascular system, both ET_A and ET_B receptors are present on smooth muscle cells [337] however the majority are of ET_A type [338]; whereas endothelial cells express solely ET_B receptors [335]. The ET_A receptor couples with Gαq, Gα11, Gα12/13 Gαs and Gαi3 proteins, signaling for vasoconstriction and smooth muscle proliferation [339, 340] whereas ET_B couples with Gαq, Gα11, Gα12/13, Gαi1 and Gαi2 G-proteins [339, 341, 342]. Activation of ET_A and ET_B receptors on vascular smooth muscle results in increased intracellular calcium and constriction via Gαq-phospholipase C pathway [343]. In most but not all vessels, stimulation of endothelial ET_B receptors leads to vasodilation through the endothelial release of prostacyclin and nitric oxide as well as opening of K^+ channels [344, 345]. The majority of circulating endothelin binds to and is cleared by by ET_B receptors in the lungs; thus ET_B receptors also prevent overstimulation of ET_A receptors [346, 347]. Further, in rat pulmonary

endothelial cells, ET-1 stimulation of ET_B receptors decreases endothelin-converting enzyme expression, suggesting a role for ET_B receptors in the downregulation of ET-1 secretion [348].

I. Endothelin signalling in PPHN

In isolated lungs from seven-day-old piglets, a low dose ET-1 infusion results in vasodilation, which is sensitive to NO blockade; however a higher dose infusion causes prolonged vasoconstriction [349]. A similar biphasic response is also observed in ovine lungs [350]. In 2-weeks old piglets, treatment with the non-selective ET receptor antagonist bosentan during an acute hypoxic exposure prevents the increase in PVR and pulmonary artery pressure; post reoxygenation, treated animals showed an improved alveolar-artery gradient, PVR and pulmonary pressure compared to controls [351]. Biphasic responses or age-dependent responses may be attributed to the variable developmental regulation and expression of ET_A or ET_B receptors in the pulmonary vasculature of a given species. In late gestation fetal lambs, pulmonary artery and inferior vena cava injection of ET-1 causes vasodilation with increased flow and this vasodilation is partly sensitive to K_{ATP} channel blockade [352]. In newborn lambs, a pulmonary artery infusion of ET-1 does not change pulmonary artery pressure in control animals, but stimulates a decrease in pulmonary artery pressure via ET_B receptor stimulation in hypoxia- induced and thromboxane-induced pulmonary hypertensive newborn lambs, via generation of NO and opening of K_{ATP} channels [353, 354], suggesting a counter-regulatory role for ET-1 in PPHN animals, presumably via endothelial ET_B signaling. While blockade of ET_B receptors does not change basal pulmonary tone in late gestation fetal lambs, antagonism of ET_A receptors and agonism of ET_B receptor stimulates potent pulmonary vasodilation, suggesting that a primary effect of endogenous ET-1 is pulmonary vasoconstriction in late pregnancy [352, 355]. Pulmonary vasodilation at birth in lambs is reduced in presence of ET_B receptor blockade [356].

Partial compression of the ductus arteriosus increases circulating ET-1, which reduces pulmonary endothelial nitric oxide synthase by increasing hydrogen peroxide (H_2O_2) production in pulmonary smooth muscle cells [357]. Increased ET-1 levels after partial ligation of ductus arteriosus is responsible for reduced angiogenesis via activation of Rho kinase [358] and decreased expression and activity of PPARγ [359]. Fetal lamb duct ligation followed by chronic ET_A antagonist treatment results in low PVR and diminished right ventricular hypertrophy as well as attenuated muscularization in the distal pulmonary arteries of delivered lambs [360]. On the other hand, chronic but not acute ET_B receptor antagonism in late gestation fetal lambs results in pulmonary hypertension with right ventricular hypertrophy and distal pulmonary artery remodeling, producing a PPHN phenotype similar to that produced by antenatal duct ligation [361]. In newborn rat PASMCs exposed to 60 per cent oxygen, ET-1 promotes remodeling via inhibition of apoptosis induced by paclitaxel and serum deprivation [362].

In summary, it appears that the major effect of endogenous ET-1 is vasoconstriction in the late gestation pulmonary circuit. However exogenous ET-1 has a biphasic effect on the fetal pulmonary vasculature. Higher levels of ET-1 are implicated in the pathophysiology of PPHN by impairing angiogenesis in endothelial cells, enhancing vasoconstriction and remodeling of pulmonary smooth muscle cells via decreasing PPARγ signalling, increasing Rho kinase activation and generation of reactive oxygen species. Endothelin receptor antagonism may attenuate these effects, with careful attention to ET_A- versus ET_B-mediated effects.

1.3 Regulation of smooth muscle calcium

Smooth muscle cells line the walls and tubes of various organs such as stomach, intestine, bladder, airways and blood vessels. In these organs contraction and relaxation of smooth muscle changes the diameter of the organ, resulting in mixing and movement of the food, emptying of the bladder, and regulation of blood flow to the various organs [363]. Smooth muscle cells in general lack the characteristic striations present in skeleton and cardiac muscles and unlike skeletal muscle cells, smooth muscle cells are not under voluntary control [363]. Mechanically, smooth muscles are of two types: i) phasic and ii) tonic. Phasic smooth muscle cells are fast contracting; examples of phasic smooth muscle include bladder and intestine. These smooth muscles display rapid rates of force activation, have increased actomyosin adenosine triphosphatase (ATPase) activity and a rapid velocity of shortening; whereas tonic smooth muscle, such as vascular smooth muscle maintains a basal tone, displays a slow rate of force activation and relaxation, slow velocity of shortening and relatively lower actomyosin ATPase activity [364].

The resting membrane potential (Em) of vascular smooth muscle cells is determined by concentration gradients as well as relative permeability of K^+, Na^+, and Cl^- ions across the cell membrane [365]. The cell membrane is relatively more permeable to K^+ than to Na^+ and Cl^-. Further, the plasma membrane Na^+-K^+- ATPase pump actively transfers 3 Na^+ ions out, and 2 K^+ ions into the cell; together this mechanism give the cell interior a net negative charge compared to extracellular side [365]. Since a vascular smooth muscle cell also has some basal permeability for Na^+ and Cl^- ions, the resting membrane potential is much less negative (-40mV to -60mV) than the equilibrium potential for K^+, which is -83mV [365]. Due to relative ion permeability and a definitive gradient across the cell membrane, K^+ channels are the major determinants of resting

membrane potential [365, 366]. These are of 4 types: Voltage-dependent K^+ channels (K_v) channels, Calcium-activated K^+ channels (K_{ca}), Inward rectifier channels and two pore domain K^+ channels [366]. Calcium levels in the vascular smooth muscle cytoplasm are kept under strict control at a physiological concentration of 0.1μM [367]. To achieve this, calcium is actively pumped out of the cell as well as sequestered inside cellular organelles [367]. Calcium is actively pumped out by the plasma membrane Ca^{2+}-ATPase pump, whereas the Na^+-Ca^{2+} ion exchanger across plasma membrane uses energy stored in the Na^+ gradient to pump Ca^{2+} out while allowing Na^+ to remain inside the cell [368]. To prevent excess cytoplasmic calcium concentrations, the ER and mitochondria store calcium through energy-dependent Ca^{2+} uptake by sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) and mitochondrial calcium uniporter complexes respectively [369].

1.3.1 G-protein coupled receptors

A variety of ligands ranging from photons, hormones, neurotransmitters, lipid mediators and sensory stimuli interact with specific proteins on cell membranes with seven trans membrane-spanning α helices, known as seven trans-membrane receptors or G-protein-coupled receptors (GPCRs). These proteins undergo coupling with various heterotrimeric GTP-binding proteins to initiate signal transduction within the cell [370]. In humans approximately 800 genes encode GPCRs [371]. GPCRs play a very significant role in the physiology and pathophysiology of several diseases [372], and agonists or antagonists targeting GPCRs account for about 36 percent of current pharmaceutical drugs [373].

1.3.2 Heterotrimeric G-protein interaction with GPCRs

G-proteins are normally present in a heterotrimeric form (Gα, Gβ and Gγ), in which guanosine di phosphate (GDP)-bound Gα reversibly associates with Gβγ proteins [374]. Gβγ binding not only helps in membrane anchorage of the heterotrimer, but also prevents premature GDP release from Gα proteins [375]. The conventional model of heterotrimeric G-protein activation states that GPCR stimulation of a cell allows the G-protein heterotrimer to contact with cytoplasmic loops of the stimulated GPCR, causing conformational changes in Gα subunit, which then stimulates GDP release from the Gα subunit [376, 377]. GDP release from Gα transiently increases affinity between GPCR and G-protein; however release of GDP is immediately followed by new GTP binding to Gα subunits [376]. GPCRs thus act as guanine nucleotide exchange factors for Gα subunits [377]. GTP binding to the Gα subunit decreases affinity between Gα and Gβγ proteins, and this promotes dissociation of heteromer into GTP-bound Gα and Gβγ protiens, which may now interact with various effectors such as phospholipase C, Rho guanine nucleotide exchange factors (Rho GEFs), and ion channels to propagate signal transduction [378].

1.3.3 GPCR desensitization

GPCR desensitization is characterized by loss of subsequent agonist responsiveness following an initial agonist stimulation [379]. GPCR desensitization can be of two types: homologous desensitization, and heterologous desensitization [379]. In homologous desensitization, the agonist-occupied GPCR is phosphorylated on intracellular serine or threonine residues by the action of G-protein coupled receptor kinases (GRKs) or other kinases such as PKA, protein kinase C (PKC), protein kinase G (PKG) which are activated by the same agonist bound to the GPCR [379]. GPCR phosphorylation by GRKs promotes arrestin binding on the receptor regions which participate in G-protein coupling, and thus arrestin binding decreases GPCR- G-protein

binding. This is followed by GPCR internalization via formation of a clathrin-coated pit [380]. Formation of a clathrin-coated pit involves binding of β-arrestin to the protein β2-adaptin, which interacts with plasma membrane via phosphatidylinositol 4,5 bis phosphate, while recruiting assembly of required proteins such as clathrin, dynamin and EGFR protein tyrosine kinase substrate 15 (EPS-15), and driving polymerization of clathrin to form a pit containing the agonist-bound receptor and arrestins [381]. Finally, dynamin, a guanosine triphosphatase (GTPase), drives invagination and scission of the coated pit from the membrane, releasing the vesicle into the cytoplasm [382]. Internalized GPCRs, after agonist removal and dephosphorylation, are either degraded or trafficked back to the plasma membrane for another round of agonist stimulation [380]. In contrast to homologous desensitization, heterologous desensitization is a generalized mechanism of GPCR desensitization in which various kinase enzymes (other than GRKs) phosphorylate one or other GPCRs, irrespective of their agonist-bound state [379].

1.3.4 Canonical signal termination mechanisms

GTP-bound Gα proteins and Gβγ dimer are very active signaling proteins and can continue to propagate signal if not returned to their GDP-bound heterotrimeric form [383]. To prevent continued stimulation of GPCRs and thus uncontrolled signal amplification, termination of GPCRs and G-protein signaling in the cells is achieved by two major mechanisms: GPCR desensitization followed by subsequent internalization; and switching of active GTP-bound Gα to its inactive GDP-bound state [384]. Gα subunits have intrinsic GTPase activity that hydrolyzes the bound GTP to return to the inactive GDP-bound state; but this intrinsic GTPase activity is very slow and cannot account for the rapid termination of signaling observed in living cells [375]. Cells have GTPase activating proteins called RGS (regulator of G-protein signaling)

proteins, which increase the intrinsic GTPase activity of Gα subunits by several fold [384]. RGS proteins are characterized by having an RGS domain that interacts with GTP-bound Gα subunits to increase their intrinsic GTPase activity, thus accelerating the formation of inactive GDP-bound heterotrimer Gαβγ, ready for another round of activation [378] **(Figure 1.2).**

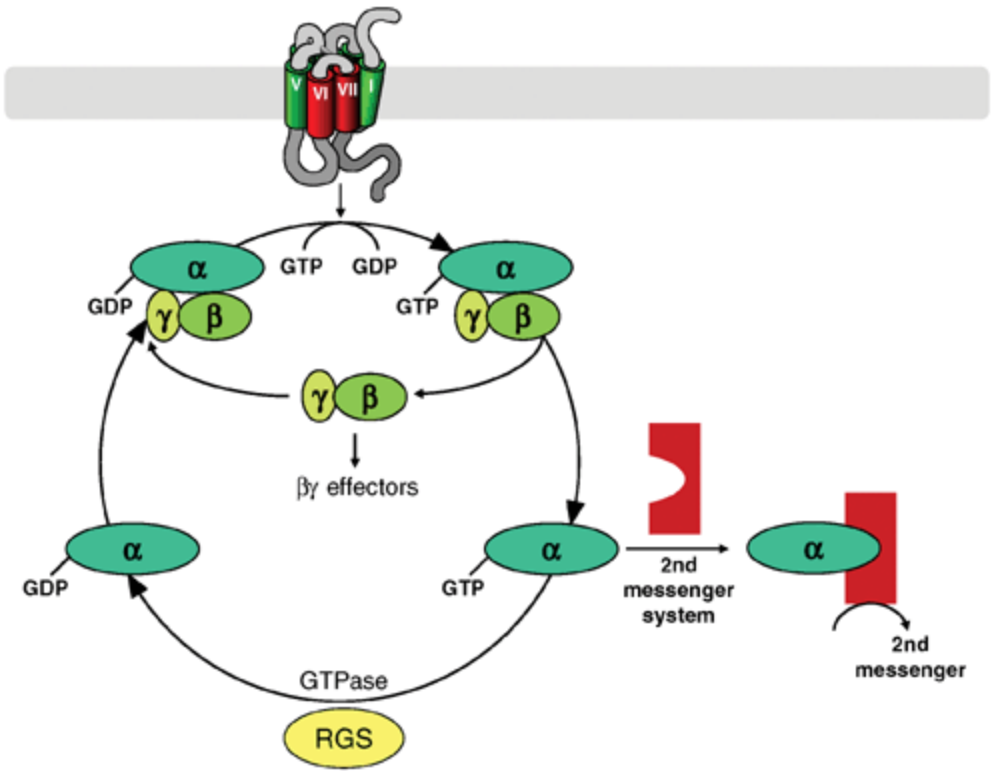


Figure 1.2 G-protein cycle

The nature of the G-protein *α*-subunit-bound nucleotide controls the extent and temporal kinetics of G-protein signaling. Conversion of a G-protein heterotrimer from the inactive, GDP-bound, to active GTP-bound state is promoted by interaction with a guanine nucleotide exchange factor (GEF), the most common of which are members of the GPCR family. Subsequent conformational changes promote separation of the GTP- bound *α*-subunit from the *β*/*γ* complex, whereupon both elements of the G-protein can regulate the activity of effector proteins that include second messenger generating enzymes and ion channels. The intrinsic GTPase activity of the G-protein *α*-subunit hydrolyses the terminal phosphate of bound GTP and terminates function. This activity is accelerated by GTPase-activating proteins, the largest family of which are the regulators of G-protein signalling (RGS) proteins. Reassociation of G*α*-GDP with the *β*/*γ* complex terminates effector regulation by the *β*/*γ*-subunits and completes the cycle. Further interaction with a GEF is now required to reinitiate the cycle.

1.3.5 Calcium mobilization in smooth muscle

Contraction of smooth muscle cells can happen in two ways. The first is electromechanical coupling, in which a depolarizing signal, arising from various stimuli such as nerve signals or stretch, initiates membrane depolarization that increases the open probability of calcium channels, leading to an increase in cytosolic calcium concentration up to 1μM [385]. High-voltage-activated L-type and low-voltage-activated T-type calcium channels are the major calcium channels taking part in depolarization-induced calcium entry in the vasculature [386]. The second mechanism is pharmaco-mechanical coupling, which utilizes mainly the activation of various G-protein coupled receptors by endogenous ligands to raise intracellular calcium, without apparent changes in membrane potential [387]. Smooth muscle cells express a host of GPCRs, which upon activation by endogenous or synthetic ligands will activate downstream G-proteins mainly $G\alpha_q$ and $G\alpha_{12/13}$. This activation initiates events triggering calcium mobilization from intracellular stores and calcium sensitization of contractile apparatus [388]. GPCR activation of G-protein Gαq activates enzyme phospholipase Cβ that generates the second messengers IP_3 and diacyl glycerol (DAG) from phosphoinositol 4,5 bisphosphate on the membrane [388]. IP_3 is a water-soluble molecule which diffuses into the cytoplasm and activates endoplasmic reticulum (ER)-localized ion channels called IP_3 receptors, to release ER stored calcium into the cytosol [134]. Calcium release from ER further opens more IP_3 channels and also activates ER-localized ryanodine receptors, releasing more calcium in the cytoplasm [389]. IP_3 and ryanodine receptors thus take part in calcium-induced calcium release [390]. DAG is a hydrophobic molecule, which stays on the membrane after generation, and alone or together with calcium ions, activates the PKC enzyme [391]. Increase in intracellular calcium is associated with vascular smooth muscle contraction, proliferation and migration [365]. In smooth muscle

contraction, cellular calcium ions interact with the calcium binding protein known as calmodulin to form a Ca-calmodulin complex [392]. Ca^{2+}-calmodulin complex and PKC activate the myosine light chain kinase (MLCK) enzyme [392]. Activated MLCK transfers phosphate from adenosine triphosphate (ATP) to Ser19 of the 20kDa regulatory myosin light chain, thus increasing its ATPase activity; this initiates cross bridge cycling by ATP hydrolysis [392]. Myosine light chain phosphatase (MLCP) then dephosphorylates myosin light chain, making it ready for another round of phosphorylation by MLCK [393].

GPCR activation of $G\alpha_{12/13}$ proteins mediate translocation of Ras-homologous-guanine nucleotide exchange factors (Rho-GEFs) such as p115-Rho-GEF, PDZ-Rho-GEF, leukemia-associated Rho-GEF (LARG) from cytosol to cellular membrane [394] whereas activation of $G\alpha q$ activates translocation of p63-RhoGEF [395]. Rho-GEFs serves two functions; first they activate Rho A, a small G-protein with GTPase activity, by facilitating the exchange of GTP over GDP on RhoA and second they terminate signal propagation by increasing GTPase activity of $G_{12/13}$ proteins via their RGS homology domain [394]. Active RhoA activates Rho kinase enzymes that inhibit MLCP enzyme by phosphorylating threonine residues of myosine phosphatase target subunit 1(MYPT1) subunit of MLCP [396]. Rho kinase and PKC independently activate an MLCP inhibitor called C-kinase-activated protein phosphatase-1 inhibitor (CPI-17) that acts as a pseudo-substrate for MLCP slowing myosin light chain (MLC) dephosphorylation leading to sensitization of the contractile apparatus [388]. Increase in polymerized actin is another mechanism by which Rho kinase contributes to vasoconstriction and this is achieved by activation of LIM-kinase followed by inhibition of severing protein cofilin by LIM-kinase [397]. Thus, Rho kinase can augment vasoconstriction by various mechanisms such as inhibition of MLCP and induce actin polymerization without further

changes in given intracellular calcium levels, and this phenomenon is termed as calcium sensitization [388].

Finally, calcium may gain entry into vascular smooth muscle via receptor operated calcium channels (ROC) and store-operated calcium channels (SOC), both of which can have overlapping triggers [398]. The main action of ROC and SOC is to replete intracellular calcium stores which have been mobilized and exhausted by pharmacomechanical coupling. Activation of various GPCRs and receptor tyrosine kinases generates DAG and activates PKC, which can stimulate calcium entry via ROC [399]. Calcium entry through SOC is triggered by depletion of intracellular calcium reserves, of which ER calcium depletion is a major stimulus [399]. A variety of plasma membrane/ER proteins take part in ROC and SOC activation, including orai 1, canonical transient receptor potential channels (TRPC), stromal interaction molecule 1(STIM1), sodium calcium exchanger, and acid-sensing ion channel 1(ASIC 1) [399, 400]. Depletion of ER calcium reserve triggers a conformational change, oligomerization and translocation of STIM1 proteins to ER-plasma membrane junction to form a channel with plasma membrane localized orai1 and TRPC1 proteins separately, which allow calcium entry into the cytoplasm[365]. TRPC provides a non-selective influx of cations in a voltage-independent manner, and there are seven members in the TRPC family TRPC1-7 [401]. Of these, TRPC3, 4,6 and 7 can be directly activated by DAG [401] and thus take part in ROC whereas TRPC1, 4, 5 and also TRPC3 and TRPC7 take part in SOC [398]. In caveolae of smooth muscle cells, entry of Na^{+} from TRPC channels and ER calcium depletion stimulates Na^{+}-Ca^{++} exchanger into reverse mode, which extrudes Na^{+} out while it transports Ca^{++} inside the cell [365]. Another ion channel involved in store depletion-induced calcium entry is ASIC1. ASIC1 is a non-selective cation channel that is activated by release of calcium from ER and allows the influx of Na^{+} and Ca^{2+} ions with more

permeability for Na^+ ions [400]. The local rise in Na^+ through ASIC1 induces Na^+-Ca^{2+} exchanger in reverse mode causing an increase in calcium entry into the cell [400].

In conclusion, smooth muscle calcium levels are tightly regulated by the interplay between various selective and non-selective ion channels, which control entry of extracellular calcium into the cell. GPCR-activation of G-proteins such as Gαq leads to calcium mobilization from the ER whereas activation of Gα12/13 stimulates calcium sensitization of the contractile apparatus. Depletion of calcium stores in ER activates further calcium entry via various proteins and ion channels such as STIM, Orai, TRPC, and ASIC1. Normal levels of intracellular calcium are achieved back by filling of ER stores as well as out flux of excess calcium by membrane-localized Ca^{2+} pumps.

1.4 Posttranslational modifications of G-protiens and prostanoid (TP, IP) receptors

1.4.1 Lipid modifications

Lipid modifications of proteins are of four types: glypiation, cholesteroylation, prenylation and fatty acylation [402]. Of these lipid modifications, fatty acylation and prenylation play vital roles in trafficking, cellular localization, protein-protein interactions and signal transduction through heterotrimeric G-proteins and G-protein coupled receptors [403]. In the process of fatty acylation, fatty acids are covalently attached to particular amino acids in proteins by various enzymes. A majority of proteins in the cell are covalently modified by two fatty acids; 14-C myristate and 15-C palmitate; hence these fatty acylations are also called myristoylation and palmitoylation respectively [404]. Glycosylation and phosphorylation are other post-translational modifications that are involved in GPCR traffic to the membrane, ligand binding, as well as the regulation of downstream signal propagation and termination [405]. A brief description of the role of lipid modifications, phosphorylation, and glycosylation in heterotrimeric G-protein and prostanoid receptor trafficking and signal transduction is presented below.

I. Prenylation

Prenylation is an enzymatic non-reversible lipid modification of proteins in which 15-carbon farnesyl or 20-carbon geranylgeranyl moieties are covalently linked via thioether linkage to candidate cysteine residues on proteins [406]. Enzymes which catalyze prenylation exist in dimer form in the cytoplasm, and are of three types: farnesyl transferase (FTase), geranylgeranyltransferase (GGTase) 1 and GGTase 2 [407]. FTase transfers a farnesyl group from farnesyl pyrophosphate to the cysteine residue of –CAAX motif of proteins, where C is cysteine, A is any aliphatic amino acid and X is any other amino acid except leucine or

isoleucine. However, if X is leucine or isoleucine, then cysteine of –CAAX motif is geranylgeranylated by GGTase-I [408, 409]. The only known targets for GGTase-2 are the Ras-related proteins in brain (Rab) family of small G-proteins with consensus sequence –CC or –CXC [410]. GGTase 2, together with a Rab escort protein, attaches two geranylgeranyl groups to the proteins of Rab family small G-proteins [411]. In proteins bearing a -CAAX sequence, any prenylation is usually followed by proteolysis of the last three amino acids by a Ras and a-factor converting enzyme 1(RCE1), followed by reversible carboxymethylation of the cysteine carboxyl group in the ER by isoprenylcysteine carboxymethyltransferase (ICMT) enzyme [412]. Among Rab family GTPases, proteins with –CXC sequence undergo carboxymethylation, while proteins with –CC sequences do not [413]. Carboxymethylation of the terminal prenyl cysteine further increases the hydrophobicity of the protein and increases membrane attachment [414]. Prenylation directs protein localization and function by acting as a hydrophobic anchor and plays a role in protein-protein interactions [404].

II. Myristoylation

Myristoylation is a permanent lipid modification, which includes co- and post-translational stable attachment of 14C fatty acid myristate via an amide bond to free N-terminal glycine residues by the enzyme N-myristoyltransferase (NMT) [415]. Myristoylation is preceded by co- or post-translational removal of N-terminal methionine residue by methionine aminopeptidase enzyme, which exposes the glycine residue to be modified by N-myristoyltransferase [402]. NMT exists in two forms, NMT1 and NMT2 [416]. Besides having a free N-terminal glycine, myristoylated proteins usually have the presence of serine, threonine or cysteine residues at the sixth position from the N-terminus [402, 417].

III. **Palmitoylation**

Protein S-palmitoylation is a dynamic and reversible post-translational lipid modification during which 16-carbon palmitate moieties, either one or more, are enzymatically attached to cysteine residues via a thioester bond [418]. Palmitoylation can occur either at the N- or C-terminus of proteins and depending on the nature of the bond between cysteine and palmitate, two different types of palmitoylation have been characterized: N-palmitoylation and S-palmitoylation. Proteins are N-palmitoylated when palmitate is linked via an amide bond to cysteines whereas in S-palmitoylation, the palmitate moiety is linked through a thioester bond to cysteines. GPCRs and their α subunits are categorized under S-palmitoylated proteins [419]. G-protein α is unique in that it is both S-palmitoylated at cysteine and N-palmitoylated via a stable amide bond at N terminal glycine [420]. Palmitoylation targets proteins to the membrane and facilitates membrane insertion by increasing hydrophobicity of proteins and partitioning them into lipid rafts [421]. Covalent and reversible addition of palmitate on specific cysteine residues is mediated by a unique set of 23 enzymes known as Aspartate-Histidine-Histidine-Cysteine (DHHC) palmitoyl acyl transferases (PAT) that contain a conserved DHHC cysteine rich domain essential for PAT activity. PATs are membrane proteins that reside mainly on the endoplasmic reticulum (ER) and the Golgi apparatus [422], with the exception of DHHC 5 and 20 that are present on the plasma membrane. Most PAT subtypes are expressed in the lung; however, to our knowledge, their cell specific distribution is currently unknown [423]. GPCRs are palmitoylated in the Golgi apparatus or ER-Golgi intermediate compartment [418] and roughly 80% of GPCRs have at least one palmitoylable cysteine in their C-terminus [424]. Depalmitoylation is also an enzymatic process which can occur anywhere in the cytoplasm, mediated by the cytosolic acyl protein thioestarase 1 (APT1) enzyme [425].

1.4.2 Lipid modifications of heterotrimeric G-proteins

Heterotrimeric G-proteins comprises three different subunits designated as Gα (41 kDa), Gβ (35 kDa), and Gγ (8-10 kDa) [374]. G-protein alpha subunits fall into into four classes on the basis of sequence identity and functional similarity: Gαi, Gαs, Gαq and Gα12 [426]. Lipid modifications of Gα subunits include palmitoylation and myristoylation, whereas Gγ subunits are exclusively prenylated [427]. Sites of G-protein modifications are given in the **table 1.1**. Lipid modification(s) of Gβ subunits have not been reported. Lipid modifications play a significant role in cellular signaling events by facilitating protein transport, anchoring proteins to cellular membranes and helping proteins to interact with other lipidated or non-lipidated proteins [428].

Table 1.1 Sites of G-protein lipid modifications[a]

Gα subunits	N-termini of α subunits	lipid modification
α_{i1}	M**G*C***TLSAEDKAAVERSKMID-	myristoylation, palmitoylation
α_{o1}	M**G*C***TLSAEERAALERSKAIE-	myristoylation, palmitoylation
α_{Z}	M**G*C***RQSSEEKEAARRSRRID-	myristoylation, palmitoylation
α_{t}	M**G**AGASAEEKHSREL-	myristoylation
α_{s}	M**G*C***LGNSKTEDQRNEEDAQR-	palmitoylation
α_{q}	MTLESIMA***CC***LSEEAKEARR-	palmitoylation
α_{14}	MAG***CCC***LSAEEKESQRISAE-	palmitoylation
α_{16}	MARSLRWR***CC***PW***C***LTEDEKA-	palmitoylation
α_{12}	MSGVVRTLSR***C***LLPAEAGAR-	palmitoylation
α_{13}	MADFLPSRSVLSV***C***FPG***C***VL-	palmitoylation
Gγ subunits	**C-termini of γ subunits**	**lipid modification**
γ_1	-KGIPEDKNPFKELKGGC↓VIS	farnesylation
γ_2	-TPVPASENPFREKKFFC↓AIL	geranylgeranylation

[a]The N-terminal sequences of several Gα and the C-terminal sequences of two Gγ are shown. Myristate links through an amide bond to an N-terminal glycine after the removal of the initiating methionine as indicated by G. Palmitate attaches via a thioester bond to cysteine (in bold and italics) residues in the N-terminus of Gα. γ1 and γ2 are isoprenylated through a thioether bond to a cysteine, indicated by C. After isoprenylation, the C-terminal three amino acids are removed (↓), and the new C-terminus is carboxyl methylated. This is a representative listing of G protein subunits. In humans, 16 genes encode Gα (plus additional splice variants), 5 genes encode Gβ (Gβ proteins are not known to be lipid-modified), and 12 genes encode Gγ.

1.4.3 Prenylation of Gγ proteins

Among heterotrimeric G-proteins, only the Gγ subunit undergos prenylation [427]. Of the 12 Gγ subunits, $G\gamma_1$, $G\gamma_9$ and $G\gamma_{11}$ are farnesylated; the rest of the Gγ subunits are geranylgeranylated [429]. Prenylation of Gγ subunits is not an absolute requirement for Gβγ dimer formation, however maturation of prenylated Gγ subunits precedes Gβγ dimer formation, and prenylation of Gγ is required for Gβγ transport to cell membrane, required for the binding of this complex with Gα subunits as well as interaction with Gβγ effectors such as adenylyl cyclase and phospholipase Cβ2 [427, 430].

1.4.4 Myristoylation and palmitoylation of $G\alpha_i$ family

The Gαi family members include Gαi (Gαi1, Gαi2, Gαi3), Gαo (Gαo1, Gαo2), Gαz, Gαt and Gαgust [431]. Gαi family members except Gαt and Gαgust undergo two different lipid modification events at their N-terminus [432]. The first is myristoylation on glycine two after cleavage of the initiator methionine; and the second is palmitoylation on adjacent cysteine 3 [433, 434]. Gαt is only myristoylated [435], and lipid modifications of Gαgust are not reported yet. Since most of the published studies investigated myristoylation and palmitoylation of Gαi proteins together, both myristoylation and palmitoylation of Gαi are discussed together below.

In monkey kidney COS cells, G-proteins such as Gαi1, Gαi2, Gαi3, Gαo and Gαz were shown to incorporate ^{3}[H]myristate to their N-terminal glycine residues [433]. Transfection of COS cells with a myristoylation-resistant mutant of Gαi (Gαi G2A, where glycine (G) 2 of Gαi is replaced by alanine (A)) causes localization of mutant Gαi proteins primarily in the soluble fraction, but without loss of their capacity to interact with βγ proteins [436]. Further, the non-myristoylated Gαi1 shows increased palmitate turnover compared to wild-type Gαi1[437].

Site-directed mutagenesis of palmitoylable cysteine 3 to alanine (C3A) in Gαi1 causes loss of membrane attachment of mutant Gαi1, however, membrane anchorage of Gαi1 (C3A) can be restored by co-transfection with βγ subunits [437].

Inhibition of Gαi1 palmitoylation by mutagenesis does not interfere with Gαi1 myristoylation [438], however there are contradictory reports regarding whether myristoylation of Gαi1 is mandatory for its palmitoylation. A study done in COS-7 cells suggests that prior myristoylation of Gαi1 is essential for palmitoylation to occur [438] whereas findings from another group indicate that in the presence of coexpressed βγ, non myristolated Gαi1 can incorporate ^{3}H-palmitate, suggesting that prior myristoylation of Gαi1is not obligatory for Gαi1 palmitoylation [437]. Moreover, chemical inhibition of myristoylation of Gαi1 with 2-hydroxymyristate also does not inhibit its palmitoylation and membrane association [439]. Besides targeting Gαi proteins to membrane, myristoylation and palmitoylation also play important roles in the partitioning of Gαi proteins into caveolae [440]. The non myristoylated (G2A) and non palmitoylated (C3A) mutant of Gαi partition poorly into caveolae, while palmitoylation on top of myristoylation increases caveolar targeting of Gαi proteins [440]. Regarding the functional effects of myristoylation on Gαi1 signaling, it has been shown that non myristoylated Gαi1 fails to inhibit adenylyl cyclase 5 enzyme in Sf9 cell membrane preparations [441]. Constitutively active Gαi2 mutant (Q205L) shows marked transforming effects in Rat 1a fibroblasts, however mutation of the myristoylation site in this protein abolishes the persistent inhibition of adenylyl cyclase and activation of p42-mitogen activated protein kinase by the constitutively active mutant Gαi2, without affecting membrane localization, suggesting the role of Gαi2 myristoylation goes beyond membrane anchorage [442]. A palmitoylation-resistant as well as

pertussis toxin resistant mutant (C3S/C351G) of Gαi partitions into cytosolic as well as membrane fractions [443]. Further, overexpression of this mutant Gαi with Gβγ proteins increases its partitioning into membrane fractions [443]. However, this mutant fails to couple effectively with agonist-occupied α_{2A} –adrenoceptors, suggesting the key role of Gαi palmitoylation lies in receptor G-protein coupling [443]. Generally for Gαi proteins prior myristoylation is required for palmitoylation. However, in the absence of myristoylation, co-expressed Gβγ subunits can provide sufficient membrane anchorage for Gαi by promoting its palmitoylation. Myristoylation of Gαi plays a role in its interaction with receptor as well as with downstream effectors such as adenylyl cyclase and mitogen-activated protein kinase. Finally, the role of agonist stimulation on Gαi palmitoylation is studied in CHO cells stably transfected with 5HT1a receptors [444]. Agonist stimulation increases palmitate exchange on Gαi proteins. 5HT1a receptor stimulation increases palmitate exchange onto Gαi in a dose and time-dependent manner [444]. In pituitary cell cultures, gonadotropin-releasing hormone (GnRH) stimulation increases radiolabelled palmitate incorporation into Gαi proteins [445].

Other members of Gαi family include Gαo, Gαz which are myristoylated and palmitoylated and Gαt which undergo only myristoylation [431]. Gαo is the most abundant G-protein in the brain [431] and like Gαi proteins is myristoylated at glycine two at N-terminus. Myristoylation of Gαo is necessary for membrane localization as well as the formation of a heterotrimer with βγ proteins [446]. Prior myristoylation of Gαo is required for subsequent palmitoylation of Gαo however palmitoylation of non-myristoylated Gαo is restored in the presence of overexpressed Gβγ [447].

In the retina, G-protein transducin (Gαt), normally present in rod outer segments, is myristoylated [435]. In transgenic mice having myristoylation resistant Gαt (Gαt G2A), the localization of Gαt G2A shifts towards rod inner segments with reduced GTPase activity, without affecting its interaction with βγ proteins [448].

G-protein Gαz is predominantly expressed in platelets, adrenal gland, and brain [449]. Studies done in human embryonic kidney (HEK) 293 and Chinese hamster ovary (CHO) cells suggest that wild-type Gαz is predominantly membrane bound, whereas the myristoylation-resistant mutant of Gαz (Gαz-G2A) is mainly cytosolic and incorporates little palmitate upon metabolic labeling [450, 451]. Myristoylation is important for signaling functions of Gαz, as the Gαz-G2A mutant demonstrates reduced adenylyl cyclase inhibition; it also does not show MAPK activation compared to wild-type Gαz following stimulation with D2 dopamine receptor agonist quinpirole. However, basal signaling through Gαz remains unaffected by loss of myristoylation [451, 452]. Non-palmitoylated, but myristoylated Gαz shows normal signaling functions such as inhibition of adenylyl cyclase as well as activation of MAPK following dopamine D2 receptor stimulation [451]. Inhibition of Gαz palmitoylation by site-directed mutagenesis does not affect myristoylation of Gαz. However non-palmitoylated Gαz presents itself in both membrane and cytosolic fractions with enrichment in cytosolic fractions, suggesting that myristoylation provides Gαz a non-specific membrane anchor [451]. Further, mutation of palmitoylable cysteine in Gαz increases constitutive inhibition of Gαz- mediated adenylyl cyclase but activates MAPK only in the presence of agonist [451, 452]. Palmitoylation of Gαz does not affect its intrinsic GTPase activity. However palmitoylated Gαz has less affinity for its specific GAP protein Gαz GAP and also decreases GAP activity of Gαz GAP [453]. Pulse-chase metabolic

labeling studies in CHO cells suggest that depalmitoylation of Gαz is slow, and agonist stimulation does not further increase its depalmitoylation [451].

In summary, for Gαz proteins, inhibition of myristoylation also causes a severe decrease in Gαz palmitoylation, loss of signaling functions and considerable loss of membrane attachment. In contrast, inhibition of palmitoylation does not inhibit myristoylation, and agonist-stimulated signaling through Gαz is preserved. However, inhibition of palmitoylation is associated with a random distribution of Gαz proteins in the cell, with a majority in cytosolic compartments. Gαz palmitoylation inhibits GAP activity Gαz-GAP. Wild-type Gαz exhibits slow depalmitoylation that remains unchanged following agonist stimulation.

1.4.5 Palmitoylation of Gαq family

Gαq family members include $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{14}$, and $G\alpha_{15/16}$. Of these, $G\alpha_q$, $G\alpha_{11}$, G_{12}, and G_{13} are palmitoylated at their amino terminal cysteine residues [434]. Endogenous Gα subunits ($G\alpha_q$, $G\alpha_{11}$) palmitoylation was first detected in PC12 and NG108-15 mouse/rat hybrid cells by metabolic labeling using ^{3}H-palmitate [454]. Heterologous transfection studies in HEK293 cells using wild type $G\alpha_q$, $G\alpha_{11}$ and their recombinant palmitoylable cysteine to serine single and double substitutions followed by metabolic labeling with ^{3}H-palmitate suggested palmitoylation sites on $G\alpha_q$ and $G\alpha_{11}$ to be cysteine 9 and 10 [455, 456]. Palmitoylation on cysteine 9 and 10 of these G-proteins has been shown to be essential for efficient membrane binding as well as for receptor-stimulated phosphoinositide production. Cysteine (C) to serine (S) mutants of $G\alpha_q$ and $G\alpha_{11}$ such as C9S, C10S, and C9, 10S were soluble compared to wild type $G\alpha_q$ which was entirely particulate following transfection into HEK293 cells [455]. The role of palmitoylation in membrane association of $G\alpha_q$ is controversial. Studies from Wedegaertner

et al suggest that palmitoylation of $G\alpha_q$ is mandatory for membrane recruitment [455] as the palmitoylation null mutant of Gαq (Gαq-C9,10A) was totally soluble. However others have reported that palmitoylation is not required for membrane association of both $G\alpha_q$, and $G\alpha_{11}$ as Gαq-C9,10A can be fractionated into particulate fractions [457, 458]. Regarding the effect of palmitoylation on downstream signaling, coexpression of α_2 -adrenoreceptor with $G\alpha_q$ mutant (Gαq-C9,10S) in HEK293 cells causes negligible inositol production following stimulation by α_2 –adrenoreceptor agonist UK-14304 compared to wild type $G\alpha_q$, whereas inositol production in the presence of Gαq single palmitoylated mutants (Gαq-C9S and Gαq-C10S) is normal [455]. Similar results were observed for wild type $G\alpha_{11}$ and its palmitoylable cysteine-to-serine mutants, with respect to their ability to stimulate inositol production following thyrotrophin stimulated hormone (TSH) challenge of TSH receptor [459]. The effect of agonist challenge on receptor palmitoylation state is variable. In rat aortic membranes, phenylephrine (α1-adrenoceptor agonist) stimulates palmitoylation of Gαq [460]. On the other hand, in aortic membranes from norepinephrine-infused rats, basal as well as phenylephrine-stimulated Gαq palmitoylation is decreased as compared to Gαq from saline infused rats [461]. In pituitary cell cultures, GnRH stimulation dose-dependently increases radiolabelled palmitate incorporation into Gαq/11 proteins [462].

In summary, Gαq and Gα11 are palmitoylated at cysteine 9 and 10; palmitoylation at these sites is important for signaling functions as well as for plasma membrane targeting of these Gα proteins.

1.4.6 Palmitoylation of $G\alpha_s$ family

Among other G-proteins, Gαs is unique in palmitoylation status as it is both irreversibly and reversibly palmitoylated on amino terminal glycine two via an amide linked bond and a labile thioester bond on cysteine 3 [420, 463]. Palmitoylation on glycine two promotes cysteine three palmitoylation in Gαs, increasing the overall hydrophobicity of the protein [420]. Initial work on Gαs palmitoylation revealed that a non-palmitoylated mutant of Gαs (Gαs-C3S) was predominantly cytosolic following transfection into HEK293 cells [455], and this mutation reduced cAMP generation following α_2 –adrenoceptor agonist UK-14304 stimulation, compared to wild type Gαs [455]. However, others found that a similar mutant of Gαs (Gαs-C3A) remains particulate in COS cells [464] and even the deletion of 26 amino terminal residues (delta 3-28) does not affect membrane localization of mutant Gαs compared to wild type [465]. Dynamics of Gαs palmitoylation in COS cells suggest that the $t_{1/2}$ for palmitate turnover on Gαs is around 50 minutes, which is much shorter than the $t_{1/2}$ for Gαs protein (640 minutes). Moreover, pulse-chase studies in COS cells suggest that both isoproterenol stimulation of beta-adrenergic receptor and cholera toxin treatment increase the palmitoylation of Gαs; and that this increase is due to increased turnover of the palmitoyl moiety on Gαs [464, 466]. The stoichiometry of Gαs palmitoylation observed in COS and S49 lymphoma cells indicates that the majority of Gαs remains palmitoylated within the cytoplasm, and receptor stimulation causes palmitate turnover on Gαs without changing the total amount of palmitoylated Gαs [467]. The fate of Gαs localization following receptor stimulation has been studied extensively. In S49 cyc^- cells which genetically lack endogenous Gαs, transfection of a wild type recombinant Gαs followed by isoproterenol stimulation caused 20% of Gαs to detach from membrane to cytosol [468].

Similarly in S49 lymphoma cells, around 50% of total cellular Gαs was found in the supernatant fraction after isoproterenol treatment [469]. This activation-induced trafficking of Gαs from membrane to cytosol is consistent with reported changes in Gαs cytoplasmic and membrane fluorescence after isoproterenol stimulation in mouse parotid and supra mandibular glands [470]. Isoproterenol stimulation also decreased Gαs membrane fluorescence, suggesting localization of Gαs from membrane to cytosol [470]. In the same line, exogenously expressed β2 –AR stimulation caused movement of Gαs in mouse oocyte from membrane to cytoplasm [471] The mechanism of Gαs movement from membrane to cytosol following receptor activation was studied by Wedegaertner *et al,* who based on their experiments in S49 cells, suggested that following Gαs activation, GTP-induced conformational changes trigger Gαs depalmitoylation. This makes Gαs less hydrophobic and directs it to the cytosol, making this process a part of the signal termination mechanism [472]. A pathophysiological relevance of this mechanism is seen in the disease pseudohypoparathyroidism type 1a, a neonatal diarrhea which results from loss of function mutation of Gαs due to alanine-valine-aspartate-threonine (AVDT) amino acids repeats in a region which interacts with guanine ring of GDP/GTP [473, 474]. Gαs-AVDT is constitutively active and localizes mainly to membranes of intestinal epithelial cell (IEC) lines IEC6 (rat) and DIF12 (mouse); this localization turns 50% cytosolic when these cells lines are co-transfected with Gαs/ Gαs-AVDT and depalmitoylation enzyme acyl protein thioesterase 1 (APT1), suggesting that inhibition of depalmitoylation in these cell lines is partly responsible for Gαs membrane localization [474]. In contrast to above reports, studies from MA104 cells suggest that Gαs and $G\alpha_i$ proteins concentrate within subdomains of the membrane and do not

release into cytoplasm, neither after activation with GTPγS nor following depalmitoylation of Gαs and $G\alpha_i$ with APT1[475].

Taken together, at present there are opposing reports regarding the role of Gαs palmitoylation in membrane anchorage. Receptor stimulation, as well as direct stimulation of Gαs, increases palmitate turnover on Gαs. Receptor stimulation is associated with the movement of Gαs from membrane to cytosol in most cell systems.

1.4.7 Palmitoylation of Gα12 family

Palmitoylation of G-proteins Gα12 and Gα13 was reported in Sf-21 cells using metabolic labeling with ^{3}[H]-palmitic acid [476]. Later, cysteine 11 at the N-terminus was shown to be palmitoylation site in Gα12 in insect Sf9 cells [477]. The cysteine 11-to-serine mutant of Gα12 ($G\alpha_{12}$-C11S) was able to co-immunoprecipitate like wild type Gα12 with βγ subunits in *Spodoptera frugiperda* (Sf9) cells, suggesting palmitoylation was not involved in Gα12 interactions with βγ. When $G\alpha_{12}$-C11S is transfected alone, the majority of it is localized to cytoplasm; however in the presence of coexpressed Gβγ, Gα12 retains its membrane location, suggesting that Gβγ can help target non-palmitoylated Gα12 to the cell membrane [477, 478]. Besides this, palmitoylation of Gα12 is essential for thrombin receptor coupling with Gα12, as thrombin receptor-stimulated binding of $G\alpha_{12}$-C11S with [^{35}S] GTPγS is decreased compared to wild-type Gα12 [477]. While Gαs is reported to have fast palmitate turnover and more complete depalmitoylation, palmitate turnover on Gα12 is slow and results in limited depalmitoylation of Gα12 [472, 477]. Palmitoylation of Gα12 also plays a role in the constitutive transforming activity of the Gα12 mutant Gα12-Q229L, as inhibition of palmitoylation is associated with loss

of constitutive transforming activity [478]. Palmitoylation and heat shock protein (Hsp90) both are required to target Gα12, but not Gα13, to lipid rafts [479].

Gα13 is palmitoylated on N-terminal cysteines 14, 18 and 37, as identified in infected insect Sf9 cells [480]. However, in HEK293 cells, cysteine 14 and 18 are the only palmitoylation sites; palmitoylation at these sites plays essential role for membrane anchorage of Gα13[481]. In Sf9 insect cells membranes, PAR1 thrombin receptor agonist-stimulated ^{35}[S] guanosine 5'-O-3-thiotriphosphate (^{35}[S] GTPγS) binding with cysteine-to-serine mutants of Gα13 (C14S, C18S, and C37S) was severely reduced as compared to wild-type Gα13 even in the presence of co-transfected Gβγ proteins [480]. Moreover, mutating palmitoylable cysteines 14 and 18 to serine residues, in a GTPase-deficient constitutively active mutant of Gα13(Gα13-Q226L), decreases transforming activity in Rat-1 cells and actin stress fiber formation in Swiss 3T3 fibroblast, whereas mutating cysteine 37 in $G\alpha_{13}$ QL does not change its transforming activity [480], indicating cysteine 14 and 18 are the critical palmitoylation sites on Gα13. Both cysteine residues 14 and 18 of Gα13 are essential for incorporation of radiolabelled palmitate; mutation of both the palmitoylable cysteines results in equal distribution of Gα13 in particulate and soluble fractions [481]. Palmitoylation of Gα13 is also essential for translocation of the Gα13 specific effector p115-RhoGEF from the cytoplasm to the membrane, and downstream Rho signaling by both wild-type Gα13 and the constitutively active mutant of Gα13, Gα13-Q226L [481]. However, non-palmitoylated Gα13 can also interact with p115-RhoGEF suggesting that palmitoylation of Gα13 is necessary for Gα13 specific localization but not for effector interaction [481].

In conclusion, palmitoylation plays a role in membrane targeting as well as raft association of Gα12, is important for receptor-dependent and independent signaling through Gα12, but not required for interaction with Gβγ proteins, which can target non-palmitoylated Gα12 to membranes. In contrast to Gαs proteins, palmitate turnover on Gα12 is slow. For Gα13, palmitoylation helps in membrane binding and is essential for downstream Rho signaling.

1.4.8 Role of Gβγ proteins in Gα protein lipid modifications and trafficking

In the presence of Gβγ overexpression, wild-type Gαz membrane expression is increased. In the presence of Gβγ, the non-myristoylated Gαz mutant (Gαz-G2A) not only incorporates palmitate label, but also attains normal membrane expression and function [451]. Wild-type Gαz proteins undergo slow basal and agonist-stimulated depalmitoylation, however, in the presence of overexpressed βγ, basal and agonist- stimulated depalmitoylation of Gαz-G2A is increased compared to wild-type Gαz, suggesting that when appended to Gαz, myristate is involved in the stability of palmitate [451]. If Gβγ proteins are sequestered in CHO cells, membrane targeting of wild-type Gαz and Gαz-C3A is reduced, suggesting Gβγ play an active role in trafficking of Gαz proteins [482]. For non-myristoylated G-proteins such as Gαq and Gαs, mutation of Gβγ contact sites inhibits palmitoylation and membrane binding; however, insertion of a new myristoylation site in Gαq, even while having a mutation on Gβγ binding sites to inhibit interaction with Gβγ, restores palmitoylation as well as membrane binding of Gαq [483].

1.4.9 Interplay between lipid modifications and other mechanisms in membrane anchorage of G-proteins

The two-signal hypothesis is proposed to explain the role of lipid modifications and other supportive mechanisms in membrane anchorage of G-proteins [484].The two-signal hypothesis was originally given to explain the membrane attachment of Ras proteins, which require prenylation as the first signal, and palmitoylation as the second signal, for effective membrane binding [484]. According to this model, at least two signals are required for proper attachment of G-proteins on the cytoplasmic side of the membrane. The first signal provides non-specific membrane attachment, and the second signal targets the protein into a specific membrane compartment [485]. For the Gαi family of G-proteins, the first signal is myristoylation and second signal is palmitoylation [417]. In the absence of the first signal i.e. myristoylation, Gαi family members are not efficiently membrane bound because in the absence of myristoylation, subsequent palmitoylation is blocked. However, in the absence of myristoylation, coexpressed Gβγ can work as a first signal and can promote subsequent palmitoylation (second signal) and thereby normal membrane binding [437, 485]. For Gαi family members who are only myristoylated (for example Gαt), the second signal is provided by the farnesylated Gγ protein in the Gβγ dimer [417]. Gαq, Gαs and Gα12 family members utilize N-terminal polybasic residues [486] as well as interactions with Gβγ proteins [483] as the first signal that promotes subsequent palmitoylation (second signal) on N-terminal cysteine residues to achieve normal membrane binding. The interaction between these signaling subunits at the membrane thus appears to require sequential lipid modifications, resulting in efficient and targeted membrane trafficking of each subunit.

1.4.10 Heterotrimeric G-protein phosphorylation

This review deals with the regulation of G protein signaling by G protein phosphorylation.

I. Phosphorylation of Gαi Family

The PKC enzyme is known to phosphorylate Gαi family proteins [487]. Treatment of platelets or S49 lymphoma cells with PKC activator phorbol ester results in PKC-mediated phosphorylation of GαI, and results in the reduced ability of Gαi to inhibit adenylyl cyclase following hormonal activation [487]. However, PKC is not able to phosphorylate Gαi when Gαi is bound to Gβγ proteins [487]. Phosphorylation of Gαi occurs as a result of receptor agonist interaction. Glucagon, vasopressin, and angiotensin II stimulation of hepatocytes results in phosphorylation of Gαi2 protein via either PKC and PKA, on serine residues 144 and 207 respectively. This phosphorylation correlates with reduced ability of Gαi2 to inhibit adenylyl cyclase enzyme [488, 489]. Treatment of hepatocytes with angiotensin II, arginine vasopressin, or the PKC activator PMA causes phosphorylation of Gαi2 proteins [490], whereas treatment of hepatocytes with insulin decreases basal and PMA-stimulated phosphorylation of Gαi proteins [491]. Phosphorylation of Gαi serves to limit receptor-mediated signaling, and is associated with low signaling states. Expression of Gαi proteins decreases in hepatocytes of streptozotocin-induced diabetic rats, which are characterized by altered receptor expression [492]; the Gαi proteins remaining in these diabetic rat hepatocytes are present in phosphorylated form, and show decreased inhibition of adenylyl cyclase compared to normal rats [493]. Basal phosphorylation of Gαi2 G-proteins is also increased in hepatocytes of obese Zuker rats, and this is responsible for reduced ability of Gαi2 to inhibit adenylyl cyclase [490].

II. Gαz phosphorylation

Phosphorylation of Gαz also owes to PKC activity; activation of PKC by PMA treatment of NIH3T3 cells causes phosphorylation of Gαz, irrespective of whether Gαz is GDP or GTPγS-bound (although Gαz phosphorylates poorly in the heterotrimer form). This phosphorylation does not affect Gαz-mediated adenylyl cyclase inhibition, however [494]. Gαz phosphorylation does not appear to require receptor-agonist interaction, but appears specific to PKC. Stimulation of platelets with phorbol-12-myristate 13-acetate (PMA), thrombin, or U46619 causes PKC-mediated phosphorylation of GDP-bound Gαz proteins but not of other (Gαi, Gαs, Gαq) proteins [495, 496]. Mutagenesis studies in HEK293 cells confirmed serine 27 as the primary site, and serine 16 as secondary site of PKC-mediated phosphorylation on Gαz [497]. Gαz phosphorylation induced by PMA in HEK293 cells neither requires prior myristoylation nor affects membrane anchorage [450]. Furthermore, analogs of cAMP and cGMP are not able to phosphorylate Gαz proteins in platelets [497]. Besides PKC, p-21-activated protein kinase also phosphorylates Gαz on serine 16; phosphorylation at this site interferes in the interaction of Gαz with Gβγ, and inhibits GAP activity of RGS proteins; both of these effects potentiate Gαz signaling [498]. Other Gα subunits are not phosphorylated by p-21-activated protein kinase [498].

III. Phosphorylation of Gα12 family

PKC phosphorylates serine 9 and serine 38 residues on Gα12; this is observed both *in vitro* and in NIH3T3 cells treated with PMA. The phosphorylation step may be triggered by agonist challenge; in platelets stimulated separately with thrombin and U46619, G-proteins Gα12 and

Gα13 undergo PKC-mediated phosphorylation [499]. Phosphorylation of Gα12 happens in the absence of heterotrimer formation. Following phosphorylation, the affinity of Gα12 for Gβγ decreases, but guanine nucleotide binding remains unaffected [494].

IV. Phosphorylation of Gαq and Gαs family

In platelets, Gαq is not phosphorylated by PKC activator PMA, nor by cAMP and cGMP analogs [497]. PKC and PKA-mediated phosphorylation of Gαs have been demonstrated in vitro but not in the cells [500, 501]. However, in transformed Rat-1 fibroblasts, tyrosine phosphorylation of Gαq results in increased activation of phospholipase C enzyme [502]. Tyrosine kinase $pp60^{c\text{-}src}$ preferentially phosphorylates GDP-bound Gα subunits (Gαs, Gαi1, Gαi2, Gαi3, Gαo, Gαt) in membrane vesicles; phosphorylated Gαs shows a modest increase in GTPγs binding and in the rate of GTP hydrolysis [503]. Furthermore, $pp60^{c\text{-}src}$ -mediated tyrosine phosphorylation on Gαs residues Tyr 37 and Tyr 377 might be involved in interaction of GDP-bound Gαs with βγ and receptor respectively [504]. However, studies from v-src transformed NIH3T3 cells suggest that phosphorylation of Gαs tyrosine residues results in decreased coupling of Gαs with the beta-adrenergic receptor as well as with the adenylyl cyclase enzyme [505].

Taken together, G-protein alpha subunits are subject to phosphorylation events both in vitro and in the intact cell. PKC is the major kinase enzyme responsible for phosphorylation of Gαi and Gα12 family members, whereas Gαq and Gαs family members are mainly phosphorylated through $pp60^{v\text{-}src}$ tyrosine kinase. G-protein alpha subunits, in general, are poor substrates for phosphorylation in the heterotrimeric form and following phosphorylation Gα subunits lose

affinity for Gβγ proteins. The functional effect of phosphorylation is variable among G protein species, but may be involved in the pathophysiology of diseases such as diabetes and obesity. For Gαi proteins, phosphorylation results in impaired ability to inhibit adenylyl cyclase; in contrast, adenylyl cyclase inhibition via phosphorylated Gαz remains unaffected.

V. Phosphorylation of Gγ proteins

Gβγ is a tightly bound dimeric protein complex, which functions effectively as a single unit. While there is not evidence of regulatory Gβ phosphorylation, some Gγ proteins may be susceptible. PKC causes phosphorylation of N-terminal serine 1 and serine 2 of Gγ12 proteins but not of other Gγ proteins (Gγ1, Gγ2, Gγ3, Gγ7) [506]. Subsequent studies have confirmed that the phosphorylation site on Gγ12 is serine 1 [507]. Basal phosphorylation of Gγ12 is detected in rat tissues; prior treatment of rats with pertussis toxin abolishes basal phosphorylation of Gγ12 proteins in tissues (including lungs), suggesting pertussis toxin-sensitive G-proteins may play a role in regulating signaling of other G-proteins [507]. Lysophosphatidic acid (LPA) and endothelin independently and together synergistically stimulate Gγ12 phosphorylation in Swiss 3T3 cells [507]; further, LPA-induced phosphorylation of Gγ12 is pertussis toxin sensitive while pertussis toxin only partially inhibits endothelin-stimulated Gγ12 phosphorylation [507]. Receptor-mediated (following challenge with angiotensin II or arginine vasopressin) or direct activation of PKC by PMA results in phosphorylation of Gγ12 proteins in Swiss 3T3 and mouse aortic smooth muscle cells [506]. In contrast to the effects of phosphorylation on Gα subunits, phosphorylated Gβγ12 has greater affinity for Gα subunits, however; phosphorylation decreases in the heterotrimeric form with Gαo [506]. Studies in the insect Sf9 cell system suggest that phosphorylation of Gβ1γ12 increases adenosine receptor coupling with Gαi1, decreases Gαs

stimulation of type II adenylyl cyclase, but does not affect PLCβ activation [508]. Phosphorylation of Gγ12 is also essential for the direct interaction of these subunits with F-actin, which promotes motility in NIH 3T3 cells [509].

In summary, among Gγ proteins, currently only Gγ12 appears to be the target of phosphorylation. PMA-activated PKC isoforms as well as via pertussis toxin sensitive G-proteins signaling may be responsible for this phosphorylation. Phosphorylation of Gγ12 readily happens in the Gβ protein complex, rather than when in a heterotrimeric form. Phosphorylation of Gγ12 plays a role in the regulation of heterotrimeric assembly, and in Gβγ12 interaction with various effectors such as G-protein α subunits, adenylyl cyclase enzymes, and F-actin.

1.4.11 Post-translational modifications of thromboxane prostanoid (TP) receptors

I. N-linked glycosylation

N-linked glycosylation is an enzymatic co-translational/post-translational process in which the enzyme oligosaccharyltransferase mediates the transfer of an oligosaccharide moiety in the lumen of rough endoplasmic reticulum from dolichol pyrophosphate to candidate asparagine residues of a polypeptide chain [510, 511]. The consensus sequence for N-linked glycosylation is NxS/T, where N is asparagine, x can be any residue but not proline and S/T is serine or threonine[512]. N-linked glycosylation is implicated in protein folding, trafficking and quality control, and can also play a role in GPCR signaling bias [513, 514].

N-linked glycosylation was first reported at N-terminal asparagine residues 4 and 16 of the platelet thromboxane receptor [515]. Inhibition of glycosylation by tunicamycin in human erythroleukemic cells decreases TP receptor ligand (^{3}H SQ29548) binding as well as U46619-stimulated calcium mobilization [516]. Further, mutation studies of candidate glycosylated asparagine residues to glutamine residues suggest that the monoglycosylated TPα receptor traffics normally to the plasma membrane and does not alter its coupling with Gαq, but generates less IP_3 and mobilizes less calcium than does wild-type TPα following U46619 stimulation [517]. Glycosylation null TPα is retained in ER, does not immunoprecipitate with Gαq, and shows severely compromised IP_3 generation and calcium mobilization [517]. Thus, TP receptor glycosylation plays a role in cell surface expression, ligand binding, and TP-stimulated calcium mobilization.

II. Palmitoylation of TP receptors

In humans, the TP receptor is present in two isoforms; TPα and TPβ [518]. TPα isoform is not palmitoylated, as it does not have cysteine residues in its C-terminal tail [518]. However, the C-terminus of TPβ receptor has three cysteine residues at Cys347, Cys373 and Cys377, and these residues incorporate palmitate following radiolabelled palmitate labeling, suggesting that TPβ is palmitoylated [518]. Mutagenesis of C-terminal cysteine residues in TPβ does not affect surface expression of TPβ as compared to wild type TPβ [518]. The majority of intracellular TPβ species are palmitoylated at Cys347, and palmitoylation at this site is required for efficient Gαq-PLCβ signaling and agonist-induced internalization [518]; whereas palmitoylation at Cys373 and Cys377 may be required for both agonist-induced and tonic internalization [518].

III. Phosphorylation and homologous desensitization of TP receptors

TPα and TPβ isoforms undergo phosphorylation followed by desensitization in a homologous as well as in a heterologous manner Earlier studies in HEK293 cells using stably transfected TP receptors had suggested that agonist-dependent phosphorylation of TPα and TPβ involves neither PKA nor PKC enzymes, as inhibition of PKC only decreases basal phosphorylation of TP receptors [519]. However this may have been a function of cell type, as challenge with endogenous thromboxane or with the synthetic TP agonist IBOP results in phosphorylation of TPα in a PKC-dependent manner in platelets but not in HEK293 cells [520]. Later studies using HEK293 cells with stably overexpressed TP receptors suggested the role of PKA and PKC in agonist-stimulated desensitization of TP [521, 522]. Both TPα and TPβ undergo agonist-induced desensitization, partly by PKC-mediated phosphorylation of serine 145 on ICL2 [521, 522]. The primary mechanism of homologous desensitization of TPβ involves GRK2/3 activation

following phosphorylation of TP serine residues 239 and 357, at the ICL2 and C-terminal regions respectively [522]. However, for TPα homologous desensitization includes agonist-mobilized calcium-activated endothelial nitric oxide synthase triggering activation of cGMP, and subsequent phosphorylation of serine 331 and serine 337 by PKG [521]. Another study reports GRK5/6 as candidate GRKs responsible for I-BOP stimulated TPα homologous desensitization; however, specific target amino acids were not investigated in this study [523].

In sum, both TP isoforms are candidates for homologous desensitization and show PKC-sensitive basal phosphorylation. Agonist-induced phosphorylation of TPα mainly involves PKG and GRK5/6, while for TPβ, GRK2/3 are the major proteins involved in homologous desensitization.

IV. Phosphorylation and heterologous desensitization of TP receptors

In platelets as well as in HEK293 cells with stably overexpressed TP receptors, the IP agonist cicaprost and the PGD2 receptor agonist BW245C are capable of desensitizing TPα but not TPβ, via PKA-induced phosphorylation of C-terminal serine 329 [524, 525]. PKG phosphorylates and desensitizes C-terminal serine 331 of TPα but not TPβ [526]. In HEK293 cells, PGI2 and PGE2 stimulation of EP1 receptors desensitize TPα via PKC, more efficiently than TPβ. This difference is due to differing C-terminal residues of TPα and TPβ, as truncation of the C-terminus of TP isoforms after amino acid 328 resulted in equal susceptibility to EP1-mediated desensitization [527]. In renal mesangial cells, EP1 and FP-receptor stimulation desensitize both TP isoforms via PKC phosphorylation of Thr 337 (TPα) and Thr 399 (TPβ) respectively [528]. In HEK293 cells as well as in human aortic smooth muscle cells, both the NO donor SIN-1 and the IP agonist cicaprost decrease TPα- but not TPβ-stimulated RhoA activation, in a PKG (S331)

and PKA (S329)-dependent manner respectively [529]. Other studies also suggest that serine 331 of TPα is the target for PKA and PKG-mediated phosphorylation and desensitization [530, 531]. In newborn swine pulmonary artery smooth muscle cells, tonic phosphorylation keeps TP receptors in a low-affinity state; this plays a significant role in the regulation of pulmonary artery constriction [159]. Chronic hypoxic exposure of newborn swine PASMCs decreases the basal phosphorylation of TP receptors [159] by reducing PKA activity [532]; this is followed by concomitant increase in TP receptor ligand affinity, contributing to pronounced agonist-stimulated calcium mobilization [159, 161].

To sum up, heterologous desensitization of TPα in platelets, cell lines, and other tissues involves various kinase enzymes including PKC, PKG, and PKA, which phosphorylate C-terminal serine and threonine residues. In the newborn swine pulmonary circuit, PKA is responsible for heterologous desensitization of TPα. In contrast, neither PKG nor PKA appear to play a role in heterologous desensitization of TPβ, which at present is thought to involve PKC derived specifically through EP1 receptor stimulation.

1.4.12 Post-translational modifications of prostacyclin (IP) receptor

I. Glycosylation of IP receptor

The human IP receptor is glycosylated on asparagine (N) residues N7 on the N-terminus and N78 in the first extracellular loop [533]. IP receptor expressed with a N7Q mutation shows glycosylation at N78, but the N78Q mutant fails to glycosylate at N7 [534], indicating the extracellular loop glycosylation is required for glycosylation of subsequent sites. Mutation of glycosylated residues decreases plasma membrane expression of IP receptor in the following order: wild type IP > IP N7Q > IP N78Q > IP N7, 78Q [534]. Double mutant IP N7, 78Q localizes primarily at the golgi apparatus [534]. Mutation at N7Q does not affect ligand binding, but diminishes iloprost-stimulated cAMP formation; whereas N78Q mutation shows decreased ligand binding and markedly decreased iloprost-stimulated cAMP formation. The N7, 78Q double mutant does not demonstrate ligand binding nor any iloprost-stimulated cAMP formation [534].

II. Prenylation of IP receptor

Among GPCRs, prenylation was first reported in IP receptors [535]. Initial studies using overexpressed human and mouse IP receptors in HEK293 cells suggested the presence of a prenylation motif in IP receptor, and revealed that human and mouse IP receptors are farnesylated on cysteine 414 at the C-terminus [535]. Neither mutation of cysteine 414 to a serine, nor chemical inhibition of prenylation by lovastatin, can affect binding of a ligand with IP receptors; however both interfere with iloprost stimulated cAMP generation and calcium mobilization in HEK293 cells [535]. Further, a non-prenylated IP receptor fails to co-immunoprecipitate with Gαs and Gαq proteins and shows impaired agonist-stimulated

internalization, suggesting a role of prenylation in receptor-G-protein interaction as well as in desensitization [536]. In HEK293 as well as human erythroleukemic cells, treatment with (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) HMG-CoA reductase inhibitors (statins) such as atorvastatin or lovastatin, or the farnesyl transferase inhibitor R115777, decreases IP stimulated cAMP generation and relieves TPα from IP-mediated cross desensitization [537-539]. However, in platelets from human patients undergoing atorvastatin therapy, IP-stimulated cAMP generation does not fall compared to normal subjects [537]. Agonist-induced internalization of IP receptor is dependent on IP interaction with a protein known as PDE6δ [540]. Inhibition of IP prenylation by mutagenesis prevents IP internalization by interfering with IP-PDE6δ interaction; siRNA knockdown of PDE6δ abolishes cicaprost-induced IP internalization in human aortic smooth muscle cells, confirming the functional importance of this interaction [540]. In summary, studies from cell lines indicate that inhibition of IP prenylation affects downstream signal propagation as well as signal termination by affecting ligand binding, cAMP generation, and impairing both homologous and heterologous desensitization. However, platelets from humans undergoing statin therapy do not show impairment in signaling through IP receptors.

III. Palmitoylation of IP receptor

IP receptor is palmitoylated on proximal C-terminal cysteine residues C308 and C311. Mutation of C308 and C311 to serine residues neither impairs trafficking of IP receptors to cell surface nor affects IP receptor ligand binding ability [541]. Palmitoylation at C308 is an obligate requirement for IP-Gαq coupling; at least one cysteine (either C308 or C311) has to be palmitoylated for greater efficiency of IP-Gαs coupling [541]. Prior prenylation of IP receptors is not a requirement for palmitoylation [541].

IV. Phosphorylation and internalization of IP receptor

Earlier studies in HEK293 cells found that PKC but not PKA is involved in both receptor-dependent and -independent phosphorylation of IP receptor. Inhibition of PKC abolishes basal phosphorylation and impairs agonist-stimulated phosphorylation of IP receptor [533]. Cicaprost-induced homologous desensitization of the mouse IP receptor, as studied in HEK293 and murine erythroleukemia cells, appear to involve PKA-dependent phosphorylation of serine 357 [542]. Phosphorylation at serine 357 is also essential for mouse IP to couple with Gαi and Gαq proteins [542]. But in contrast to mouse IP, human IP does not couple with Gαi proteins, and human IP coupling with Gαs and Gαq proteins is neither PKA nor PKC-dependent in HEK293 cells [543]. Iloprost-stimulated desensitization of the human IP receptor does involve PKC-mediated phosphorylation of serine 328 [544]; however subsequent internalization is partly mediated by dynamin and clathrin-coated vesicles, but does not involve β-arrestin, GRKs, or PKC [545]. Agonist-stimulated IP receptor internalization is not dependent on the presence of a phosphorylatable C-terminal tail of IP receptor, but rather is mediated through IP interaction with Rab5a protein, followed by sorting into endocytotic vesicles in a dynamin-dependent manner [546]. Subsequent recycling of IP receptors is dependent on the C-terminal tail interaction with Rab11 protein, which sorts IP receptor into recycling endosomes [547]. In human fibroblasts, iloprost-induced desensitization of IP receptors is slow and does not change in the presence of PMA (PKC activator) treatment, or GRK2/5 overexpression [548]. Agonist-induced desensitization of the IP receptor is a well-recognized clinical phenomenon which rapidly limits the longer-term utility of IP agonist vasodilator therapies. In U46619 infused isolated rabbit

lungs, vasodilatory responses of iloprost are lost within three hours of stimulation, and subsequently inhaled iloprost fails to trigger further pulmonary vasodilation [549]. However, in the presence of PKC inhibition, and EP1 receptor antagonism, iloprost-induced pulmonary vasodilation increases and subsequent desensitization is delayed [549].

In summary, studies from cell lines suggest that homologous desensitization of IP receptor involves phosphorylation via the PKC enzyme, and takes place via dynamin and clathrin-coated vesicles. Rab family proteins play a role in early sorting and recycling of IP receptors. Agonist-stimulated desensitization of IP and its downstream effects such as loss of coupling with G-proteins may vary with species. In the pulmonary circuit, the EP1 receptor is implicated in heterologous desensitization of IP receptors via PKC activation.

1.4.13 S-nitrosylation

S-nitrosylation is a reversible post-translational modification of proteins in which nitrogen monoxide group is covalently attached to a thiol group on a specific cysteine residue (typically positioned between an acidic or basic amino acid) [550, 551]. In the S-nitrosylation reaction, NO is mostly generated by nitric oxide synthase enzymes; however NO can be transferred from S-nitrosoglutathione (transnitrosylation) or an S-nitrosocysteine by a transition metal [551, 552]. Denitrosylation of nitrosylated proteins is catalyzed by glutathione (GSH), which after interaction with nitrosylated proteins becomes S-nitrosoglutathione (GSNO) and leaves free –SH groups on cysteine residues. GSNO is then converted to GSH by the enzyme GSNO reductase (GSNOR). Thus, cellular levels of GSNOR indirectly controls the denitrosylation of proteins by maintaining a proper equilibrium between nitrosothiol (SNO) proteins and GSNO [553]. Another pair of proteins involved in denitrosylation reactions is the thioredoxin (Trx)/thioredoxin reductase (TrxR) system [554]. An imbalance of the cellular redox state leads to an escalation of reactive oxygen and nitrogen species such as superoxide ion and peroxynitrate, promoting S-nitrosylation due to decreased levels of reduced glutathione (GSH) [551, 555].

1.4.14 S-nitrosylation in vascular signaling

Inhibitors of GSNOR elicit vasodilatory response in mouse aorta [556] and deletion of GSNOR is associated with reduced SVR [557]. Studies in mice suggest that SNOs decrease agonist-stimulated β2-adrenergic receptor (β2-AR) internalization by S-nitrosylating the GRK2 [558]. β2-AR agonist stimulation is also involved in dissociation of endothelial nitric oxide synthase and β arrestin-2 by S-nitrosylation of β arrestin-2 followed by binding of β arrestin-2 with clathrin heavy chain [559]. In angiotensin II-induced hypertensive mice, inhibition of

thioredoxin reductase causes an increase in S-nitrosylation of cellular proteins including soluble guanylyl cyclase [560], and this is associated with decreased relaxation to acetylcholine of phenylephrine-precontracted mouse aorta [561]. In human microvascular endothelial cells, constitutive S-nitrosylation of nicotinamide adenine dinucleotide phosphate (NADPH) subunit p47phox limits superoxide production and protects the vessel from oxidative stress [562]. In contrast, elevated nitrosylation of arginase is associated with uncoupling of the endothelial nitric oxide synthase [563]. Recent studies from mutant mice indicate that S-nitrosylation of cysteine 93 on the β chain of hemoglobin is an essential requirement for normal cardiovascular function and hypoxic vasodilation of systemic arteries [564].

Taken together, S-nitrosylation plays a regulatory role in vascular smooth muscle signaling by taking part in NO-mediated relaxation and regulating GPCR endocytosis. S-nitrosylation of hemoglobin appears to play a crucial role in hypoxic vasodilation of systemic vessels.

2 Rationale and Hypothesis

The mechanism of hypoxia-induced vascular hyper-responsiveness, and the effect of hypoxia on thromboxane receptor signaling in the newborn pulmonary circuit are important to understand the interaction of inflammatory cascade activation and hypoxic pulmonary vasoconstriction. Neonatal pulmonary arterial smooth muscle cells under hypoxic conditions show hypersensitivity and hyperreactivity of the thromboxane receptor (TP), with persistent sensitization even after cessation of hypoxia [159, 161]. In hypoxic myocytes, the increase in calcium mobilization is due to a selective increase in coupling of the TP receptor to G-protein αq instead of $G\alpha_{12/13}$ [565]. The two molecules in this receptor-effector signaling partnership are regulated by differing forms of protein post-translational modification, namely palmitoylation and phosphorylation.

This thesis collects our studies on post-translational modifications of proteins comprising the thromboxane (TP) receptor-G-protein complex, and examines the effect of pulmonary arterial hypoxia on these post-translational modifications. Our overall objective was to identify points of regulation that may serve as potential pharmacological targets, to better treat hypoxic pulmonary hypertension. In the studies introduced below, we first proposed that Gαq palmitoylation could play an important role in vascular smooth muscle contraction by regulating intracellular calcium mobilization due to TPα stimulation. We then proposed to examine the mechanism for PKA-mediated phosphorylation and desensitization of the TP receptor, and identify which C-terminal serine residue of TPα is responsible for regulation of ligand affinity. We further proposed that reduced IP-stimulated cAMP generation in hypoxia could be due to reduced adenylyl cyclase activity. Our overall hypothesis is that under hypoxic conditions, increased Gαq palmitoylation

and decreased adenylyl cyclase activity may be responsible for increased TP hyperreactivity and hypersensitivity respectively.

2.1 Rationale #1: Gαq palmitoylation study

Mechanisms determining coupling of GPCRs with Gαq under pathophysiological conditions such as hypoxia are not well defined. Differing acylation patterns and turnover among the various Gα subunits contribute to their differing kinetics of membrane shuttling [566]. Gαq protein attachment to the plasma membrane and interaction with GPCRs is regulated by palmitoylation [455, 456, 567], the posttranslational enzymatic addition of 16-carbon palmitate groups onto sulfhydryl groups of N-terminal cysteine residues 9 and 10 of Gαq [455, 566]. Many GPCRs are also subject to regulatory palmitoylation of C-terminal cysteine residues[418], and receptor palmitoylation state is known to drive increased trafficking and coupling of GPCRs with their respective G-proteins [458, 568]. However, due to the absence of C-terminal cysteines, neither porcine TP nor human TPα are amenable to palmitoylation [518]. That leaves Gα subunit palmitoylation as a candidate regulatory mechanism for coupling selectivity.

Hypoxia-induced hyperreactivity of the thromboxane receptor, for which Gαq couples receptor activation to generation of its effector, is an integral component of infantile and adult pulmonary hypertension. The effects of hypoxia on Gαq palmitoylation have not been studied, nor has the role of Gαq palmitoylation in the context of hypertension. In this study, we hypothesized that palmitoylation of Gαq enhances TP-mediated calcium mobilization, exacerbating hypoxic pulmonary vasoconstriction.

Objective #1: To characterize effects of *in vitro* and *in vivo* hypoxia on Gαq palmitoylation, and its impact on TP- Gαq coupling and downstream signalling.

Hypotheses: (i) Reversible palmitoylation of Gαq is increased by hypoxia in pulmonary artery but not aortic myocytes; (ii) Hypoxia will increase TP-Gαq interaction by increasing Gαq palmitoylation; (iii) Gαq palmitoylation will cause a hyper-reactive calcium response to thromboxane; (iv) Site-directed mutagenesis of palmitoylable cysteine residues will decrease TP-stimulated calcium mobilization. (v) Hypoxia will induce pulmonary arterial hyperreactivity to thromboxane, and pharmacological inhibition of palmitoylation will decrease TP-stimulated force generation in hypoxic pulmonary artery rings with minimal effect on control pulmonary artery rings.

2.2 Rationale #2: TP phosphorylation study

Phosphorylation in response to signaling cross-talk with other prostanoid receptors or ligand binding is a major regulatory mechanism of TPα signaling [569]. Functional differences between TP isoforms reflect differing G-protein coupling, desensitization and receptor internalization following agonist stimulation [570]. The C-terminal sequence variation between TP isoforms results in different patterns of phosphorylation. TPβ, but not TPα, undergoes agonist-induced phosphorylation by GRKs, leading to receptor internalization [571], whereas the C-terminus of TPα is not capable of being phosphorylated by GRKs [523]. Desensitization of TP receptors commonly involves a decreased affinity of the receptor for its agonist and is associated with the establishment of an intermediate affinity state [572], requiring TP receptor phosphorylation [573]. TPα undergoes desensitization due to phosphorylation of the C-terminus serine residues by PKA and PKC [526, 574, 575]. The association of TPα desensitization with activation of

adenylate cyclase-coupled receptors and increased cAMP production suggests that PKA is likely to be involved in this effect [576].

We studied TP receptor hyper-reactivity in a porcine model of hypoxic PPHN. Only one porcine TP isoform has been identified; therefore, any variations in porcine TP signalling can be attributed to changes mediated by the hypoxic TP receptor, not to receptor isoform differences. Amino acid sequence alignment indicates that the porcine TP receptor is homologous with the human TPα isoform, although the number of phosphorylation-amenable serine residues is greater in porcine TP (five serines) than in human TPα (four serines) (Supplementary Figure 7.4). Gαq co-immunoprecipitates with the porcine TP receptor [565], suggesting that porcine TP signalling is also similar to that of human TPα. Previously, we showed that serine phosphorylation is decreased in TP receptors from hypoxic myocytes [159]; this difference correlates with increased Gα/q coupling and TP agonist hyper-responsiveness in hypoxia, and is prevented by incubation of myocytes with PKA [161]. We previously demonstrated that hypoxia attenuates PKA activity in pulmonary arterial myocytes due to hypoxic impairment of prostacyclin receptor activity, thereby inducing dephosphorylation and sensitization of the TP receptor agonist response [532]. However, we did not identify the serine residues responsible for the functional regulation of TP receptors. In this study, using in vivo and in vitro models, we examined agonist responses in hypoxic human and porcine TP receptors, and using site-directed mutagenesis, identified the phosphorylated serine residues that render the TP receptor desensitized in the presence of PKA activation.

Objective # 2: To determine the C-terminal serine residue of TPα responsible for PKA-mediated phosphorylation.

Hypotheses: (i) Of the available intracellular serine residues in the TPα structure, one or all of the C-terminal serines 324, 329 and 331 may be amenable to selective phosphorylation by PKA. (ii) Phosphorylation at this locus will decrease ligand binding affinity of TP.

2.3 Rationale #3: Adenylyl cyclase study

Prostacyclin stimulates IP receptors to activate the adenylyl cyclase (AC) enzyme via G-protein Gαs [577]. In the newborn swine pulmonary circuit, TPα receptors are maintained in a state of low affinity by PKA-mediated tonic phosphorylation through IP receptors [159]. In the presence of hypoxia, tonic phosphorylation of TPα is abolished resulting in TP hyper reactivity that is associated with reduced IP-stimulated cAMP generation as well as reduced PKA activity [532]. We propose that cAMP-activated PKA phosphorylation of TPα is a hypoxia-sensitive axis of TP regulation. AC is known to be activated by Gαs-coupled receptors for vasoactive and cardiotonic agents including prostacyclin, epinephrine and dobutamine, stimulating cAMP generation. We therefore propose to examine the effect of hypoxia on basal and receptor-stimulated AC activity. Cardiac inotropic and vasoconstrictor/dilator agents acting via Gαs- and Gαq-coupled receptors often have impaired biological activity in hypoxic pulmonary hypertension [578-581]. Transient neonatal hypoxia impairs the postnatal development of pulmonary artery adrenergic vasorelaxation [581], and persistently impairs cardiac AC activity and left ventricular contractility to adrenergic stimulation [582, 583], which despite adequate treatment of pulmonary hypertension, may not recover [584]. In contrast, chronic hypoxia in adults impairs

AC pathway activity only transiently, permitting full recovery of cardiac function [585]. Endogenous prostaglandins mediate nearly 70% of basal AC activity in pulmonary artery, with the majority of autocrine and paracrine production being prostacyclin [586]. As the AC-cAMP pathway plays a critical role in regulating pulmonary arterial tone, there is increasing clinical enthusiasm for use of cAMP pathway agents to treat PPHN, especially given risks of reactive nitrogen species generation during nitric oxide and oxygen therapy. A meta-analysis of inhaled iloprost (PGI_2 analog) in neonatal and pediatric pulmonary hypertension noted its acute hemodynamic effects are comparable to nitric oxide [587]. cAMP analogs have equal efficacy in intact pulmonary artery and in endothelial dysfunction [588]. Inhaled iloprost improves right ventricular dynamics in acute hypoxia [589] and may be preferable to nitric oxide for treating pulmonary hypertension in context of left heart failure [590]. Agents elevating cAMP potentiate cGMP formation, and also attenuate pulmonary artery myocyte proliferation [591], important for alleviating pulmonary arterial remodeling in PPHN patients. Despite the therapeutic importance of this pathway, and the fact that tissue hypoxia is frequently present when Gαs-stimulating agents are utilized in PPHN, the role of hypoxia in AC regulation and the specific effects of neonatal hypoxia on contemporaneous AC-mediated responses of the pulmonary circuit are not well understood. In this study, we examine effects of moderate *in vitro* hypoxia on receptor-dependent and –independent activation of AC in neonatal pulmonary artery myocytes, to determine the impact of neonatal hypoxia on prostacyclin receptor-mediated cAMP release, and on the kinetics of AC activity.

Objective # 3: To study the effect of *in vitro* and *in vivo* hypoxia on adenylyl cyclase activity and to investigate a possible mechanism for decreased AC activity.

Hypothesis: (i) Hypoxia will decrease both basal and stimulated adenylyl cyclase activity in newborn swine pulmonary artery smooth muscle cells; (ii) This decreased activity will be associated with impaired relaxation to AC-activating agents such as forskolin in pulmonary artery rings from hypoxic piglets compared to control piglets.

3 Methods

3.1 General Methods

3.1.1 Induction of hypoxic pulmonary hypertension

Newborn piglets <24 hours age obtained from a pathogen-free farm supplier were exposed to hypoxia (FiO_2 0.10) for 72 hours, maintained in a sealed plexiglass isolette with diurnal cycling and continuous flow of room air mixed with nitrogen (~8L/min), and fed swine milk replacer ad libitum. Age-matched normoxic controls were similarly raised in room air. After 72 hour environmental exposure, piglets were euthanized by pentobarbital overdose; heart and lungs harvested en bloc. Elevated pulmonary resistance was confirmed by presence of cardiac right ventricular hypertrophy [592]. This protocol was approved by the University of Manitoba Animal Ethics Board, per NIH animal ethics standards and the Canadian Council for Animal Care.

3.1.2 Primary cell culture and in vitro hypoxia

New born swine pulmonary artery and aortic smooth muscle cells were obtained using a dispersed cell culture method selective for myocytes [593]. Second to sixth generation pulmonary arteries and descending aorta from newborn swine (<24 hours old) were recovered in ice-cold HEPES-buffered saline (HBS; in mM: 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, 10 glucose; pH 7.4) with antibiotic/antimycotic, washed in Ca^{2+}-reduced HBS (20 μM $CaCl_2$), finely minced and digested for 15 min at 37˚C with type I collagenase (1750U/mL) and papain (9.5U/mL) in 1mM dithiothreitol and 2mg/mL bovine serum albumin. Dispersed cells were collected by centrifugation, washed and resuspended in culture medium, and seeded at density 4.4 x 10^4cells/cm^2 in Ham's F-12 medium with 10% fetal bovine serum, 1% penicillin

and 1% streptomycin. At 80% confluence, cells were serum-deprived for 48 hours in Ham's F-12 with L-glutamine/penicillin/streptomycin and 1% insulin transferrin-selenium to synchronize cells in a contractile phenotype, then placed into hypoxic (10% O_2) or normoxic (21% O_2) culture environment for 72 hours.

3.1.3 Culture of human PASMCs

Human primary pulmonary artery smooth muscle cells (hPASMC, passage number 2, from 21 year old male; ATCC PCS-100-023) were grown in Vascular Cell Basal Medium supplemented with 5ng/ml FGF-b, 5ng/ml EGF, 5%FBS, 5µg/ml insulin, 50µg/ml ascorbic acid, 10mM L-glutamine, 10U/ml penicillin, 10µg/ml streptomycin and 25ng/ml amphotericin B, and grown to 80% confluence before passage. After one passage, hPASMCs were grown to confluence, then serum-deprived for 2 days in Vascular Cell Basal Medium with 0.5% growth factors to synchronize in a contractile phenotype. Cells were maintained in serum-deprived media, and randomized into normoxic (21%O_2, 5%CO_2) or hypoxic environment (10%O_2, 5% CO_2) for an additional 72hrs.

3.1.4 Isometric myography

Pulmonary arteries from hypoxic, normoxic and newborn day 0 piglets were carefully microdissected into ice-cold Krebs buffer (in mM: 25 $NaHCO_3$, 112.6 NaCl, 4.7 KCl, 1.38 NaH_2PO_4, 2.46 $MgSO_4 \cdot 7H_2O$, 5.56 Dextrose; pH 7.4), then cut into 2 mm rings and mounted on pins attached to force transducer on a multi-chamber isometric wire myograph (Danish Myo Systems) in Krebs buffer at 37°C, continuously bubbled with 95% O_2 + 5% CO_2, pH 7.4-7.45. Rings were allowed to equilibrate for 45 minutes at optimal length determined from a length/ tension curve giving maximal active tension to 100mM KCl stimulation. Ring viability was

tested using KCl; all force measurements were normalized to maximal contractile response to 100mM KCl.

3.1.5 Membrane fractionation

Cells were washed with ice cold PBS and collected into buffer A (10mM Tris-HCl, pH 7.4 with protease inhibitors 1 mM EDTA, 10 μg/mL benzamidine, 10 μg/mL leupeptin, 20 μg/mL soybean trypsin inhibitor, 5 μg/mL aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride). Cell suspension was homogenized in a glass douncer on ice by giving 30 strokes followed by centrifugation of suspension at 300g for 10 min at 4˚C. The supernatant fraction was further centrifuged at 48,000g for 20min at 4˚C. The resulting pellet was resuspended in buffer B (50 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, having protease inhibitors as in Buffer A) and suspension was centrifuged again at 48000g for 20 min. Finally pellet was resuspended in buffer B and aliquots were snap frozen and stored at -80˚C. Membrane protein concentration was determined by DC protein assay from Bio-Rad.

3.1.6 Immunoprecipitation

Cells were washed free of media into ice-cold phosphate buffered saline. Cells were lysed in ice-cold lysis buffer (in mM: 50 Tris, 150 NaCl, 1 EDTA, and 1% Triton X-100, pH 7.4) for 30 minutes with vortexing every 10min. Cell lysate was centrifuged at 10,000g for 10 min to remove debris and protein concentration in clear supernatant was measured by Bradford assay. Lysates (500μg) were precleared by incubation with 50% v/v bead slurry of protein G Sepharose beads (GE Healthcare) prepared in lysis buffer. After preclearing, lysates were rotated overnight at 4˚C with antibody. The precipitate was pulled down with 30μL of 50% bead slurry followed

by washing of beads with ice cold lysis buffer and boiled in 2x Laemmli loading buffer (Bio-rad) with β-merkeptoethanol (Sigma) for Western blotting.

3.1.7 Western blotting

Whole cell lysates were collected on ice in lysis buffer as above and boiled in 2x Laemmli loading buffer (Bio-rad) with β-merkeptoethanol (Sigma) followed by protein separation using 10% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and then blocked for 1 hour at RT in Tris-buffered saline with 0.1% Tween (TBS-T) and 5% skim milk. Blocked membranes were treated overnight at 4˚C on a shaker with primary antibodies diluted in TBS-T with 1% skim milk and next day membranes were washed 5 times with TBS-T for 1 hour followed by 1 hour incubation with secondary antibody in TBS-T with 1% skim milk. Enhanced chemiluminescence (Thermo) was used to visualize protein bands. Data were quantified under non-saturating conditions, with manual background subtraction by a digital imaging densitometer.

3.1.8 Stable or transient expression of TP receptors and substitution mutants

To establish stable TP expression, wild-type porcine TP or human TPα genes containing a rho-1D4 tag [C-terminal epitope T-E-T-S-Q-V-A-P-A-(COOH) of rhodopsin] were inserted into pcDNA 3.1 and transfected into HEK293T cells (10 μg plasmid per 10 cm plate) using lipofectamine 2000. Cells were grown in DMEM with 10% FBS, 50 $U \cdot mL^{-1}$ penicillin and 50 $mg \cdot mL^{-1}$ streptomycin. After 48 h, stably expressing HTP and PTP clones were selected using 1 mg·mL−1 geneticin (Invitrogen) for 3 days. TP expression was detected by antibody to rho-1D4 **(supplemental Figure 7.1).** One clone for HTP and one for PTP was selected for Ca^{2+} mobilization assay **(supplemental Figure 7.2)** and saturation binding assay **(supplemental Figure 7.3)**. Amino acid substitutions with alanines into positions Ser239, Ser324, Ser329 or Ser331 were introduced into a synthetic human TPα gene with rho-1D4 tag, carried by the pMT4 expression vector. Transient transfection of HEK293T cells in 6-well tissue-culture-treated BD-falcon plates were transiently transfected with 10 μg per well plasmid DNA containing wild-type TPα or mutants Ser239A, Ser324A, Ser329A and Ser331A, using lipofectamine. To minimize variation in transfection efficiency, 1 μg cDNA was transfected per $7x10^5$ cells. After 12 h of transfection, media was replaced with DMEM-F12 with 5% FBS.

3.1.9 Transient expression of wild type Gαq and cysteine substitution mutants

Wild type Gαq and its cysteine to alanine substitution mutants C9A, C10A, C9AC10A were transfected into HEK293T cells as described above.

3.2 Methods (Gαq palmitoylation study)

3.2.1 Isometric myography

Pulmonary arteries (60-100μm) from hypoxic, normoxic and newborn piglets were mounted on an isometric wire myograph with palmitoylation inhibitor 10 μM 2-BP or diluent for 3 hours. Isometric force responses were measured to incremental doses of TP agonist U46619 and normalized to maximal contractile response to 100mM KCl.

3.2.2 Calcium mobilization after pharmacological inhibition of palmitoylation in primary myocytes

Hypoxic and normoxic pulmonary artery and aortic myocytes were treated with 5μM 2-bromopalmitate (palmitoylation inhibitor) or diluent for 2 hours, then loaded with 5μM Ca^{2+}sensitive fluorescent dye fura 2-acetoxymethyl ester (fura-2AM) in DMSO, with 1μg/ml pluronic acid (for AM ester solubilization) in HBSS buffer, for 1 hour at 37°C. Extracellular fura-2AM was removed by three times wash with HBSS buffer and de-esterification allowed for 30 min. Coverglass plates were secured on an inverted microscope (Olympus) in room air, and studied at 20x magnification. For TP studies, aortic and pulmonary artery myocytes were challenged with serial concentrations of U46619 (thromboxane mimetic). For purinergic receptor studies, pulmonary artery myocytes were challenged with serial concentrations of ATP in Ca^{2+} -free HBSS. Emissions at 510nm wavelength after 340nm / 380nm excitation were captured by

NIS-Elements software. Peak Ca^{2+} was measured in 8 regions per field, each containing 3-5 cells, after subtraction of background (from cell-free areas).

3.2.3 IP_3 measurement

Confluent serum-deprived hypoxic and normoxic pulmonary arterial myocytes incubated with or without 5μM 2-bromopalmitate for 2 hours, were challenged with 10^{-6} M U46619 for 1 min; unstimulated controls were used to determine basal inositol-1,4,5-trisphosphate (IP_3). Cells were lysed and intracellular IP_3 extracted with ice-cold 20% trichloracetic acid for 20 min. Precipitated proteins were sedimented by centrifugation; supernatant was neutralized using 10M KOH. Intracellular IP_3 generation was quantified using TRK1000 radiocompetition binding assay (GE Life Sciences) employing [^{3}H]-myo-IP_3 as the radioactive standard, expressed as picomol IP_3 per mg lysate protein (measured in the pelleted cellular extract).

3.2.4 Site directed mutagenesis

Genes for Gα subunits, with an internal EE-tag, were obtained from the Missouri S&T cDNA Resource center (Rolla, MO, USA). The Gαq, C9A, C10A and C9A/C10A mutations were commercially synthesized in pcDNA3.1 (Genscript).

3.2.5 Metabolic labeling

Whole cell palmitate uptake was measured by incubating pulmonary artery myocytes plated at density $4.4x10^4$ cells/cm^2 with 150μCi/ml ^{3}H-leucine(to label all new protein) and 150μCi/ml ^{14}C-palmitic acid during 72 hour hypoxic or normoxic growth. Cells were lysed into standard volumes, and protein palmitate uptake was quantified as a ratio to protein synthesis, by scintillation counting of ^{3}H and ^{14}C per unit volume lysate. To measure Gαq-specific

palmitoylation, HEK293T cells transiently transfected with wild-type Gαq DNA with internal EE-tag using Lipofectamine (Invitrogen), were labeled 8 hours post-transfection with DMEM-F12 media containing 150μCi/ml 9, 10--^{3}H-palmitic acid, 5mM sodium pyruvate and 10% dialyzed fetal bovine serum, for 24 hours in a hypoxic or normoxic environment. Following labeling, Gαq was immunoprecipitated using antibody to the EE-tag. After protein separation by electrophoresis, the gel was fixed for 30 minutes in fixing solution (10% Glacial acetic acid, 30% methanol, 60% DD water) followed by amplification with amplifier (Amersham), dried and subject to fluorography at 80˚C for one month. Cold Gαq was similarly immunoprecipitated and quantified by Western blot as a protein standard.

3.2.6 Calcium mobilization after pharmacological inhibition of palmitoylation in HEK293T cells

HEK293T cells stably expressing TPα were grown for 72 hours under normoxic or hypoxic conditions, followed by plating 10^5 cells per well in 96-well BD-falcon optilux plates, with or without 10, 50 or 100μM 2-bromopalmitate for 4 hours. Media was removed and cells incubated for 1 hour with Fluo-4NW and 77mg/ml probenecid. Ca^{2+} mobilization was measured as change in fluorescence intensity after challenge with serial concentrations of TP agonist U46619, using Flexstation-3 fluorescence plate reader (Molecular Devices) at 525nm following excitation at 494nm to generate dose–response curves after subtracting responses of mock-transfected (blank vector) cells as background.

3.2.7 Membrane fractionation

Membranes were prepared as described in general methods from HEK293T cells stably expressing human TPα and transiently transfected with wild type or substitution mutant Gαq

genes C9A, C10A and C9A/C10A. Membrane fractions and whole lysates were separated by SDS-PAGE, and blots were probed for wild-type Gαq and Gαq cysteine mutants with antibody to EE-tag.

3.2.8 Co-immunoprecipitation of thromboxane receptor with Gαq

72 hours post transfection, TPα was immunoprecipitated from HEK293T cells stably expressing TPα transiently transfected with wild-type or mutant Gαq. Blots were probed with antibody to EE tag.

3.2.9 Calcium mobilization by Gαq cysteine substitution mutants

HEK293T cells stably expressing human TPα and transiently transfected with wild type Gαq or substitution mutants C9A, C10A or C9A/C10A were grown for 24 hours in normoxic or hypoxic conditions, then transferred to 96-well BD-falcon optilux plates at 10^5 cells/well. Cells were loaded for 1 hour with Fluo-4NW, and calcium mobilization to serial concentrations of TP agonist U46619 determined as above.

3.2.10 Statistical analyses

Quantitative data were compared by Student's t test or ANOVA with Bonferroni posttest, and dose-response curves and binding kinetics were compared by nonlinear curve fit (GraphPad Prism). Data were expressed as mean± SEM; results were considered statistically significant at $P < 0.05$.

3.3 Methods (TP phosphorylation study)

3.3.1 TP immunoprecipitation

TP was immunoprecipitated from serum-deprived hypoxic and normoxic pulmonary artery smooth muscle cells. Eluted proteins were separated by SDS-PAGE, and blots were probed with mouse anti-phospho-serine antisera.

3.3.2 TP receptor GTPase activity assay

Newborn pulmonary artery myocytes in primary culture were grown in hypoxic (10% O_2, 5% CO_2) or normoxic (21% O_2, 5% CO_2) conditions for 72 h in vitro, and then challenged with buffer or 10^{-6} M TP receptor agonist U46619, with or without 10^{-5} M SQ29548 (TP antagonist), for 2 min before flash freezing. Membrane fractions obtained by centrifugation were added to a reaction mixture containing substrate GTP. The reaction was arrested by the addition of dye (Gold mix), and read at 590 nm excitation and 650 nm emission λ by plate reader. TP receptor-specific live cell GTPase activity was measured as GTP degradation to GDP and inorganic phosphate (Pi), via colorimetric assay of the dye-Pi complex. Background enzyme activity was determined in unstimulated membranes. GTPase activity was calculated from a standard curve, expressed as inorganic phosphate liberated$\cdot min^{-1}\cdot mg^{-1}$ protein.

3.3.3 PKA activity assay

HEK293T cells were incubated for 72 h in hypoxic or normoxic conditions, as described above. After 3 min of incubation with 10^{-6} M forskolin, 10^{-6} M H8, 10^{-8} M milrinone or diluent, cells were lysed on ice. PKA activity was measured by a 96-well ProFluor assay kit. PKA substrate peptide, Bisamide Rhodamine 110 (PKA R110), was pre-incubated with 25 μL ATP, then the

PKA assay reaction mixture for 30 min, and the reaction was terminated by 25 μL protease reagent in termination buffer with 25 μL stabilizing reagent. Fluorescence of non-phosphorylated substrate, which inversely correlates with PKA activity, was measured at 485 nm excitation and 530 nm emission λ, against reagent buffer blanks, using a FLUOstar Optima microplate reader. PKA activity was expressed as fold-change compared with (untreated) normoxic lysates.

3.3.4 PKC activity assay

HEK293T cells grown for 72 h in hypoxic or normoxic conditions were incubated for 3 min with $2x10^{-5}$ M PMA, 10^{-5} M Go6983 or vehicle. Cells were lysed, and PKC activity was measured using solid-phase ELISA plates coated with PKC substrate and substrate-specific detector antibody. Experiments were repeated in the presence of 10 μL H89 (PKA inhibitor) to exclude non-specific kinase activity. PKC activity was expressed as fold-change from normoxic controls.

3.3.5 Identification of potential TP phosphorylation sites

Potential phosphorylation sites on porcine and human TP receptor isoforms were identified using the online NetPhos 2.0 server, a neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences with sensitivity reported from 69 to 96% [594] **(Supplemental Figure 7.4)**.

3.3.6 Radioligand binding assays

Saturation binding kinetics were assayed in 20 μg of separated membrane protein. Samples were incubated with the TP receptor antagonist [^{3}H]-SQ29548 (0.1–50 nM) in binding buffer, with or without an excess of unlabelled agonist U46619 (10 μM) to determine non-specific binding, in 100 μL total volume for 1 h. Reactions were terminated by vacuum filtration, and membranes

were washed with ice-cold binding buffer. Filters were agitated in distilled water to release adsorbed radioisotope and then equilibrated in 5 mL CytoScint (ICN) overnight. Unbound radioisotope was also collected. Counts min^{-1} were quantified for 3 min per sample by a liquid scintillation counter. The presence of single receptor populations, with non-cooperative binding, was confirmed by linear Scatchard transformation; equilibrium dissociation constants (K_d) and maximal binding sites (B_{max}) were determined from saturation isotherms by non-linear curve fit. Competitive binding kinetics were determined by incubation of membrane fractions with 20 nM [^{3}H]-SQ29548, followed by displacement with serial concentrations of cold U46619. Unbound antagonist was removed by filtration. Data were analysed using a one-site fit method to calculate K_i (Prism 4.03; GraphPad, La Jolla, CA, USA).

3.3.7 Calcium mobilization assay

After transfections, HEK293T cells were incubated for 72 h (stable cell line) or 48 h (transient transfection) in hypoxia or normoxia, prior to calcium mobilization studies. HEK293T cells stably expressing TP receptors were incubated with 10^{-6} M forskolin, H8, PMA, Go6983 or buffer for 1 h. Cells were loaded with calcium-sensitive dye Fluo-4NW (Invitrogen). Receptor-mediated Ca^{2+} mobilization was reported as change in fluorescence intensity after being treated with 10^{-4}– 10^{-10} M U46619 (TP agonist), in a Flexstation-3 fluorescence plate reader at 525 nm emission following excitation at 494 nm, to generate dose–response curves after subtracting the responses of mock-transfected cells (negative controls, pMT4 vector only) to eliminate baseline fluorescence. EC_{50} values were calculated by non-linear regression analysis (Prism 4.03; GraphPad).

3.3.8 Statistical analyses

Normally distributed quantitative data were analysed by Student's t-test or ANOVA with Bonferroni's post test; dose–response curves and binding kinetics by non-linear curve fit. All data are expressed as mean±SEM; $P < 0.05$ was considered significant.

3.4 Methods (Adenylyl cyclase study)

3.4.1 Adenylyl cyclase activity assay

Human PASMCs (hPASMCs) and porcine PASMCs (pPASMCs) were challenged with prostacyclin mimetic 1μM iloprost, direct activation of AC with 1μM forskolin, or diluent for 30 min, then lysed. Lysates were adjusted to 5μg protein/μl, in 20mM Tris buffer with protease inhibitors. AC activity assay was carried out in 96-black-well plates, with serial wells holding 10^{-10} to 10^{-3}M ATP in buffer containing 0.25mM TerbiumIII, 0.05mM Norfloxacin, 10mM $MgCl_2$, 20μg $CaCl_2$, 20mM Tris-HCl, and 1% BSA, at 25ºC, with reaction commencing by automated addition of 250μg lysate to the reaction mixture. Fluorescence intensity of terbium norfloxacin was acquired by BMG FLUOstar OPTIMA microplate reader (Jena, Germany) using 337nm excitation and 545nm emission wavelengths with a signal integration time of 60μs, against reagent buffer blanks, with 3-8 replicates for each point. AC specific activity was determined by loss of ATP-bound terbium norfloxacin fluorescence due to ATP-to-cAMP conversion [595]. Stability of fluorescence loss at constant substrate concentration was verified; optimal assay time determined by a linear product-versus-time curve in presence of excess substrate. Finally, AC velocity was determined as a function of substrate ATP concentration, as Δfluorescence/min/mg protein, in lysates from each treatment group.

3.4.2 BAPTA treatment

Normoxic and hypoxic porcine PASMCs were lysed in 20mM Tris buffer with protease inhibitors. AC activity assay was done in the presence of 2μM calcium chelator BAPTA.

3.4.3 Pertussis toxin treatment

Hypoxic and normoxic porcine PASMCs were treated with pertussis toxin (100ng/ml for 16 hours) followed by lysate preparation and AC activity assay as described above.

3.4.4 ATP assay

Human PASMCs were homogenized in ice-cold lysis buffer (20mM Tris-HCl, 1.5mM NaCl, 0.1mM ouabain, 3mM Na azide). ATP content was immediately determined by luciferase activity, quantified by luminometer at 560nm, per manufacturer's directions (ATP kit, Molecular Probes), normalized to protein content.

3.4.5 Phosphodiesterase (PDE) activity assay

cAMP-specific PDE assay was performed using reagents from QuantiZyme Cyclic Nucleotide PDE colorimetric assay kit (Biomol), in a protocol modified to assess cAMP degradation due to lysate enzyme activity [596]. Cell lysates were collected from porcine PASMCs grown under normoxic or hypoxic conditions, in 10mM Tris-HCl buffer (pH 7.4), after 5 min incubation with 1μM vinpocetine (inhibiting PDE1; Enzo Life Sciences, Burlington, Canada), 1μM Bay-60-7550 (inhibiting PDE2; Cayman Chemical), 1μM milrinone (inhibiting PDE3), or 1μM rolipram (inhibiting PDE4). Lysates were centrifuged at 100,000x*g* to obtain crude cytosolic fractions. The assay mixture, containing 20μl of 0.5mM cAMP substrate, 75μg cytosolic protein and assay

buffer, was incubated at 30°C for 30 min. Reaction was terminated by addition of Biomol Green reagent. AMP resulting from the action of PDE was degraded by 5'-nucleotidase to release free phosphate, generating a colorimetric reaction quantified by a microplate reader at 620nm, against a blank containing cytosolic protein without substrate. Phosphate release was calculated from a linear standard curve generated using 5'AMP concentrations of 0.05-3.0nM and 5'nucleotidase, and normalized to protein content [597].

3.4.6 Reverse transcriptase (RT)-PCR

RNA was extracted from hPASMCs using Qiagen RNeasy Plus per manufacturer's instructions. RNA purity was determined by spectrophotometer. Reverse transcription and PCR reactions were carried out using One-Step RT-PCR (Qiagen). For each reaction, 100ng RNA (or water, for negative control) was used with isoform-specific forward and reverse primers (0.125μM). Primers were designed using Integrated DNA Technologies PrimerQuest Software and were blasted to ensure no sequence overlap **(Table 3.1)**. PCR amplifications were carried out using a Techne Genius Unit under conditions: reverse transcription at 50°C for 30 min, initial activation at 95°C for 15 min, 30 amplification cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 60°C, followed by 1 min extension at 72°C. Final extension was at held at 72°C for 10min. PCR products were separated by 2% agarose gel electrophoresis and visualized with GelStar. Bands were analyzed by densitometry and were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 3.1 Adenylyl cyclase primer sequence and predicted size

Gene	Forward primer	Reverse primer	Product size
ADCY1	GGTTGTTCACACCACAGGCACAAT	TATCCATGCAGTGAGAGGCAGCTT	93
ADCY2	TCTCACGATCATCACCACAGCCAT	TTTGTTGTGTTGGCAGTTGGAGGG	105
ADCY3	TCATGTGTGCTGTGGTCTTCTCCA	TGCAGAGCACGAAGAGGATGATGT	96
ADCY4	TCACGGCCAGAGAGCACTAACAAT	GGCAATCTGGTCGAACTTGCCAAA	171
ADCY5	TGAAAGCAGACATCAACGCCAAGC	CAGCAAACAGGATGCTCACGTTGT	90
ADCY6	AGACATGATTGAGGCCATCTCGCT	ACATCGAACTGCCATTTCCGCAAG	126
ADCY7	TCTTTGGACCTGCACTGGAGGATT	TGTTACAGACCAGGCACAGAGCTT	115
ADCY8	TCAGGTATGTGCGGTCAAGGACAA	CCAGCGTGGCTTTGGAAATGTGAA	192
ADCY9	AAGCATGGCCAGTGCAATTCTGAG	ATTTGTGGAAGCCAAGATGGCACC	151
GAPDH	TCGACAGTCAGCCGCATCTTCTTT	ACCAAATCCGTTGACTCCGACCTT	94

3.4.7 Prostacyclin (IP) receptor kinetics

Normoxic and hypoxic pPASMC were pretreated for 24h with 10^{-6}M U46619, 10^{-6}M iloprost, 10^{-6}M 8-bromo-cAMP or diluent. Cells were rinsed free of media, and whole cell lysates obtained by scraping cells into binding buffer (25mM Tris, 10mM $CaCl_2$, 0.01mM indomethacin and 75µg/ml PMSF, pH 7.4). Particulate matter was separated by brief centrifugation at 700*g*; the supernatant was then ultracentrifuged at 100,000x*g* for 60 min at 4°C, and the membrane fraction was resuspended in binding buffer. 30-60µg membrane protein was used for all radioligand experiments. Saturation binding kinetics were quantified in membrane fractions, by incubation with various concentrations of ^{3}H-iloprost (0.1, 1.0, 10, 50, and 100 nM) diluted in binding buffer, with or without an excess of unlabelled iloprost (10µM), in a total reaction volume of 100µl for 2 hour at 25^{0}C. Reactions were terminated by vacuum filtration, and membranes were washed twice with ice-cold binding buffer. Filters were agitated in 500µl distilled water to release adsorbed radioisotope, then equilibrated in 5ml CytoScint (ICN) for 5 hours before counting. Unbound radioisotope was also collected. Counts per minute (CPM) were analyzed for 3 min per sample.

3.4.8 Receptor immunoprecipitation and Western blot

4µg antibody to IP receptor (rabbit; Cayman Chemical) was bound to Sepharose G beads with 200µg whole cell lysate overnight at 4°C. Beads were washed and precipitated protein was eluted and boiled in Loading Buffer (Biorad). Equal volumes of immunoprecipitated protein, and equal µg of pre-cleared whole lysate, were separated by SDS-PAGE, transferred onto nitrocellulose membranes and probed with rabbit-anti-Gαi or rabbit-anti-Gαs. Blots were measured by enhanced chemiluminescence, developed on X-ray film, and bands analyzed by densitometry.

3.4.9 Isometric myography

Dose-dependent relaxations to AC activator forskolin and nitric oxide donor sodium nitroprusside were studied on pulmonary artery rings (500-600um) from 3-day normoxic and hypoxic piglets precontracted with 30nM TP agonist U46619.

3.4.10 Statistical analyses

Data analysed by unpaired t-test or one-way ANOVA with post-hoc Tukey's test; enzyme kinetics by non-linear regression, using GraphPad Prism software. Data are expressed as mean±SE; $p<0.05$ considered significant.

4 Results and Discussion

4.1 Results (Gαq palmitoylation study)

4.1.1 Effect of 2-bromopalmitate treatment on response to TP challenge of hypoxic pulmonary artery rings and pulmonary artery myocytes

Pulmonary artery rings from newborn (Day 0), 3-day-old normoxic (control), and 3-day-old hypoxic (PPHN) neonatal swine were challenged with thromboxane mimetic U46619. TP-stimulated maximal contractile force decreased from Day 0 to Day 3 of life. Pulmonary arteries from Day 3 PPHN piglets were hyperreactive to stimulation by U46619 compared with Day 0 and Day 3 control pulmonary arteries. Pretreatment with palmitoylation inhibitor 2-BP for 3 hours (10^{-5} M) decreased the response of hypoxic pulmonary arteries, with negligible effect on Day 0 and Day 3 normoxic pulmonary arteries **(Figure 4.1A)**. U46619 responses were also studied in pulmonary artery myocytes exposed for 3 days to normoxic or hypoxic growth conditions. The U46619-induced peak calcium response for normoxic myocytes was unchanged by 2-BP pretreatment. Hypoxic myocytes were hyperresponsive to U46619. As with pulmonary arterial tissues, pulmonary artery myocytes treated with 2-BP had a significantly reduced peak calcium response to challenge with 10^{-6} M or greater doses of U46619 **(Figure 4.1B).**

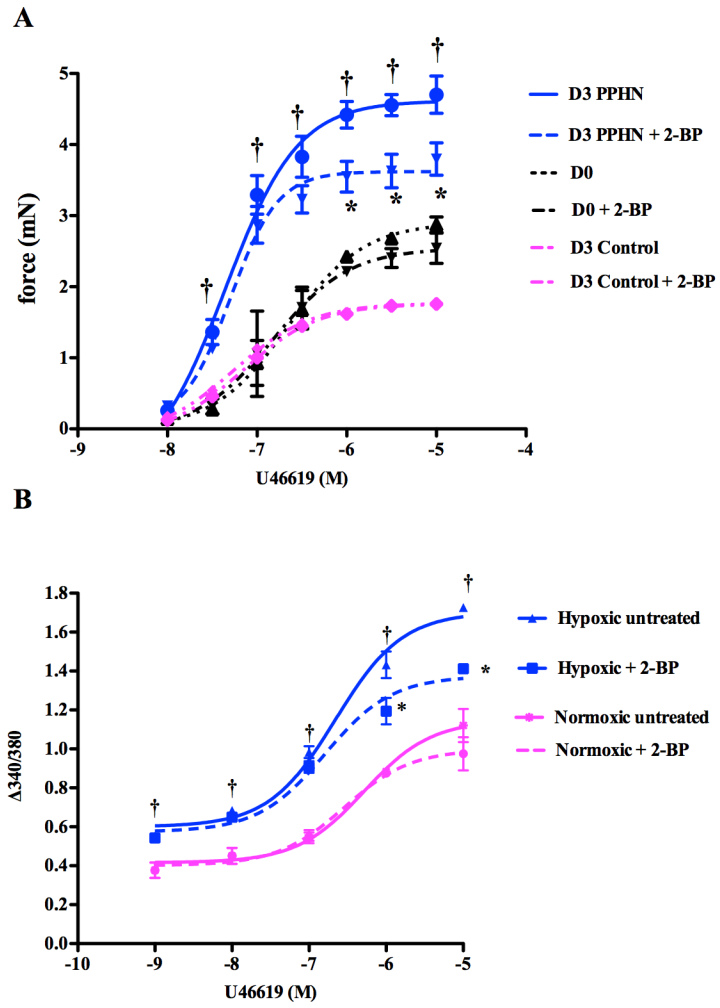


Figure 4.1 Pharmacological inhibition of palmitoylation decreases the contractile response to thromboxane prostanoid (TP) challenge of hypoxic pulmonary artery rings and pulmonary artery myocytes.

(A) Dose-dependent contraction to thromboxane mimetic U46619, measured in pulmonary artery (PA) rings from newborn (D0), 3-day normoxic control (D3), and 3-day hypoxic (D3 persistent pulmonary hypertension of the newborn [PPHN]) piglets. Maximum contractile force decreases from Day 0 to Day 3 of life ($P < 0.05$). PA rings from hypoxic PPHN animals are hyperresponsive to TP challenge compared with PA from age-matched normoxic controls (†$P < 0.001$). The protein palmitoylation inhibitor 2-bromopalmitate (2-BP; 10^{-5} M) decreases contractile force response of PPHN PA to U46619 (*$P < 0.05$) but has negligible effects on D0 and D3 Control PA. **(B)** Dose-dependent Ca^{2+} mobilization response of first-passage PA myocytes to U46619, measured after 3-day exposure to normoxia or hypoxia *in vitro*, and treatment with 2-BP or diluent for 2 hours immediately before agonist challenge. Peak Ca^{2+} assay using calciometric dye fura-2 after subtraction of agonist-naive baseline. Ca^{2+} response of hypoxic pulmonary artery myocytes differ from normoxic controls (†$P < 0.001$). 2-BP–treated hypoxic myocytes differ from untreated hypoxic cells (*$P < 0.05$).

4.1.2 Palmitoylation inhibition decreases calcium response to ATP challenge of hypoxic pulmonary artery myocytes but does not alter TP challenge of aortic myocytes

Purinergic P2Y receptors in normoxic and hypoxic pulmonary artery myocytes were stimulated with serial concentrations of ATP in a calcium-free environment.

Calcium mobilization to ATP stimulation was unchanged by hypoxic exposure. 2-BP had little effect on response to P2Y-R stimulation in normoxic cells but reduced ATP-induced peak calcium response in hypoxic cells **(Figure 4.2A)**. Aortic myocytes exhibited lower responsiveness to TP challenge than observed in pulmonary artery myocytes; responses were not clearly altered by hypoxia or 2-BP, however 2-BP treatment decreased the Ca^{2+}-response of normoxic aortic myocytes **(Figure 4.2B)**.

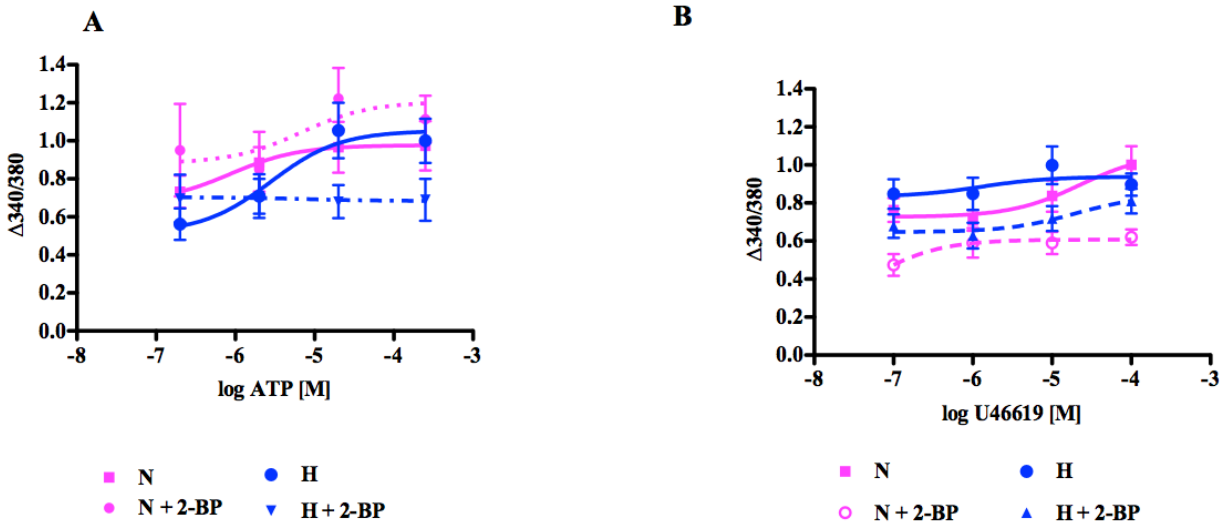


Figure 4.2 Effects of palmitoylation inhibition on calcium response to ATP challenge of hypoxic pulmonary artery myocytes and TP challenge of hypoxic aortic myocytes.

Agonist dose–response relationships of myocytes after 72 hours of normoxia (N) or hypoxia (H) treated with 2-BP ($5x10^{-6}$ M for 2 hours) before agonist challenge are shown. Peak Ca^{2+} mobilization was determined using calciometric dye fura-2 and is represented as emission ratio at 340/380λ after subtraction of agonist naive baseline. **(A)** In pulmonary artery myocytes, hypoxia has no significant effect on Ca^{2+} response of purinergic (P2X, P2Y) receptors to ATP challenge; 2-BP decreases Ca^{2+} response in hypoxic myocytes. **(B)** In aortic myocytes, Ca^{2+} response to thromboxane mimetic U46619. 2-BP slightly decreases the normoxic but not the hypoxic myocyte Ca^{2+} response to U46619 ($P< 0.05$).

4.1.3 Palmitoylation inhibition decreases TP-mediated IP_3 generation

Basal and U46619-stimulated IP_3 was increased in pulmonary artery myocytes after 72 hours of exposure to hypoxic conditions compared with normoxic controls. After treatment with 2-BP, basal and stimulated IP_3 generation dropped to levels consistent with agonist-naïve normoxic myocytes **(Figure 4.3)**.

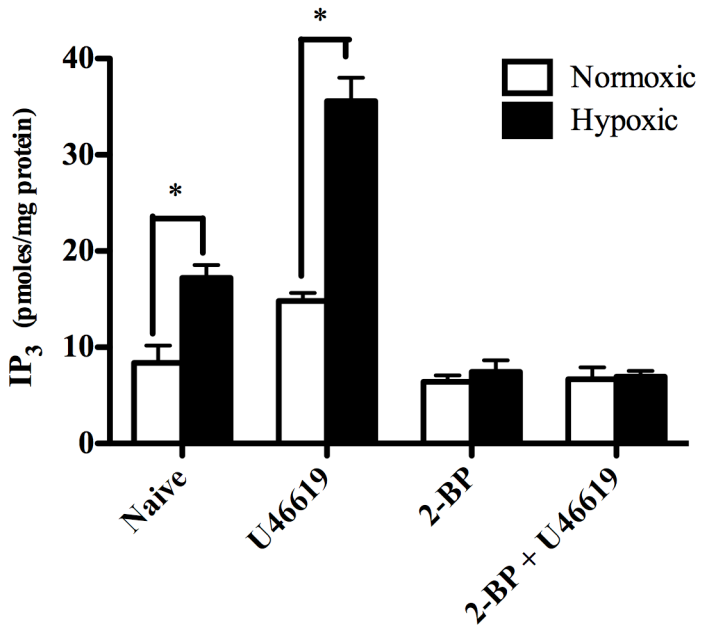


Figure 4.3 Palmitoylation inhibition decreases TP-mediated inositol-1, 4, 5-trisphosphate (IP_3) generation.

Seventy-two-hour hypoxic and normoxic pulmonary artery myocytes were treated with $5x10^{-6}$ M 2-BP or diluent for 2 hours and challenged with 10^{-6} M U46619 for 1 minute. Cells were flash-frozen, and IP_3 was quantified in whole cell lysates (pmol/mg protein). Basal and stimulated IP_3 generation was inhibited by 2-BP (n = 5) *P < 0.01.

4.1.4 Hypoxia increases Gαq palmitoylation but not palmitate uptake

Pulmonary artery myocytes labeled with ^{3}H-leucine/^{14}C-palmitate for 72 hours during normoxia or hypoxia had no difference in palmitate uptake relative to leucine uptake **(Figure 4.4A)**. Gαq-specific palmitoylation increased in HEK293T cells labeled with 9,10-^{3}H-palmitic acid for 24 hours in hypoxic conditions without change in total Gαq content **(Figure 4.4B)**.

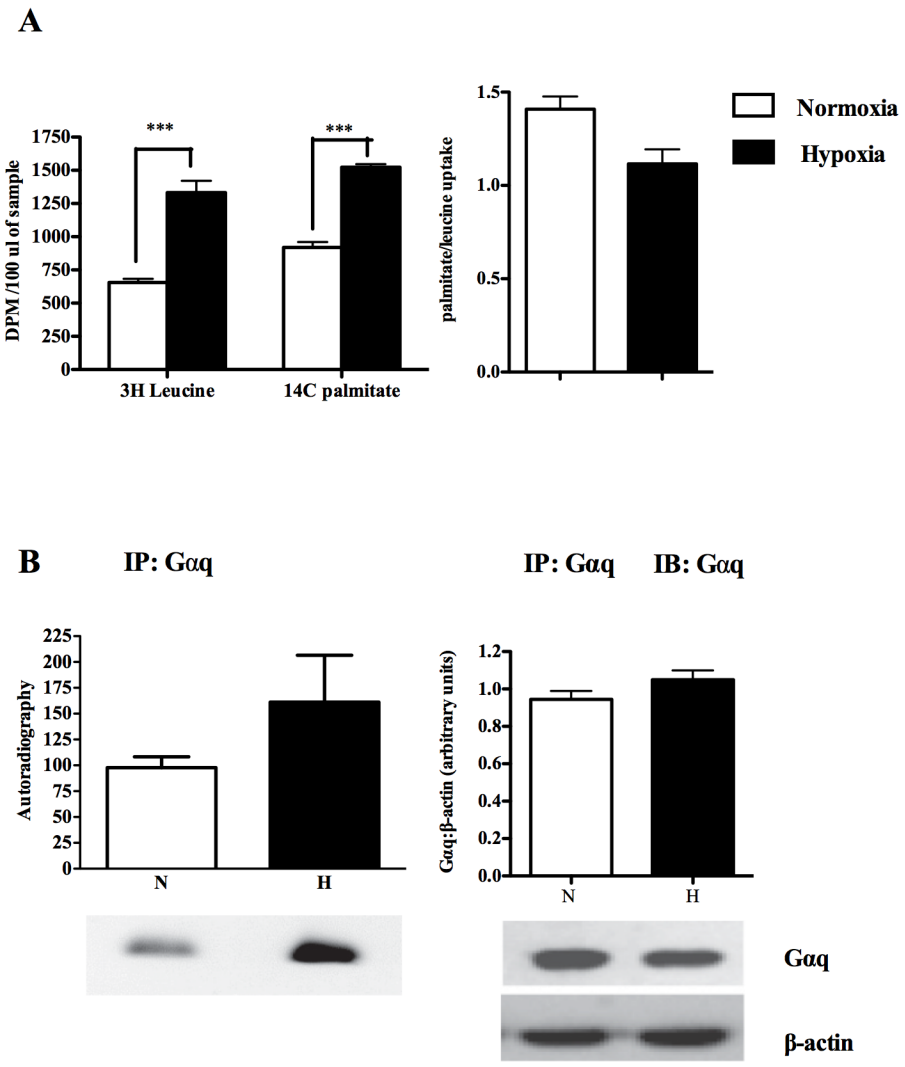


Figure 4.4 Hypoxia does not alter palmitate uptake relative to protein synthesis but increases Gαq palmitoylation.

(A) Primary pulmonary artery myocytes plated at density $4.4x10^{-4}$ cells/cm^2 and incubated with 150 μCi/ml ^{3}H-leucine and ^{14}C-palmitate during 72-hours hypoxic or normoxic growth. New protein synthesis was determined by scintillation counting ^{3}H per volume of lysate; total palmitate uptake was expressed as ^{14}C per volume. The ratio of ^{3}H to ^{14}C disintegrations per minute (DPM) represents palmitate uptake per unit protein synthesis (n = 5). ***Different from normoxic value ($P < 0.001$). **(B)** HEK293T cells transfected with EE-tagged Gαq were labeled with 150 μCi/ml 9,10-^{3}H-palmitic acid for 24 hours before lysis, immunoprecipitation of Gαq, and autoradiography of blotted proteins for 1 month. Immunoblot of cold Gαq presented as control.

4.1.5 Pharmacological inhibition of Gαq palmitoylation decreases TP-mediated calcium mobilization

U46619-stimulated calcium mobilization increased after 72 hours of exposure to hypoxic conditions in HEK293T cells stably expressing TPα compared with normoxic controls **(Figure 4.5)**. Pretreatment with low dose 2-BP (10μM) significantly decreased the calcium response of hypoxic cells with no effect on normoxic cells. Higher doses of 2-BP (50 and 100μM) dose-dependently decreased calcium mobilization but always to a greater degree in hypoxic than in normoxic cells **(Figure 4.5)**. Representative calcium traces showing the effect of 2-BP on U46619-stimulated calcium mobilization is presented in **Figure 4.6**.

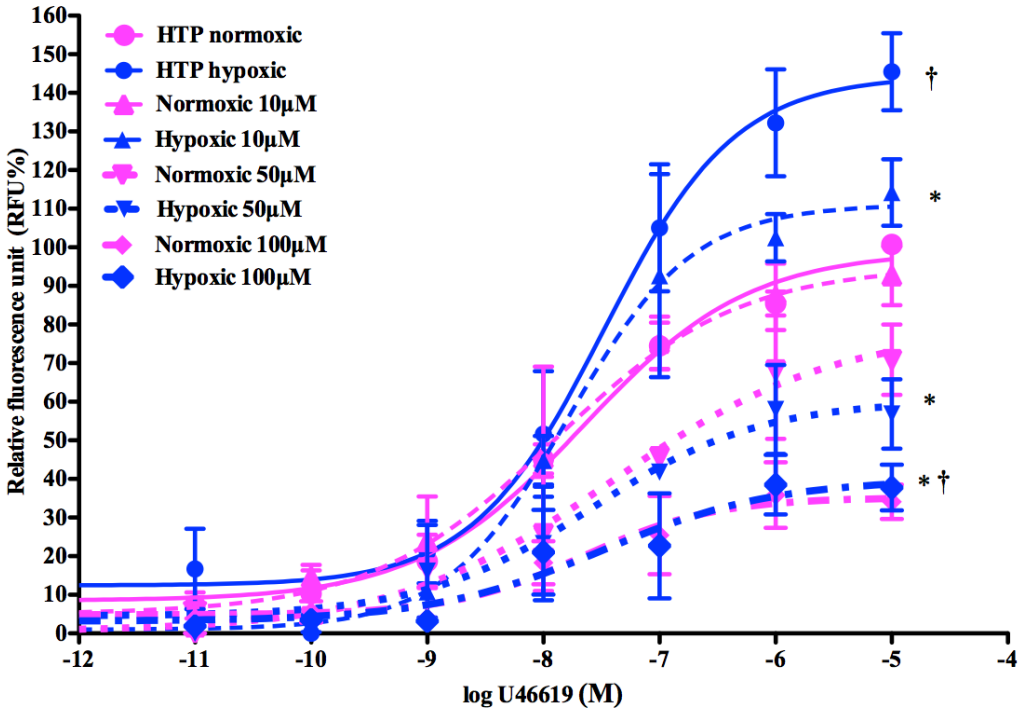
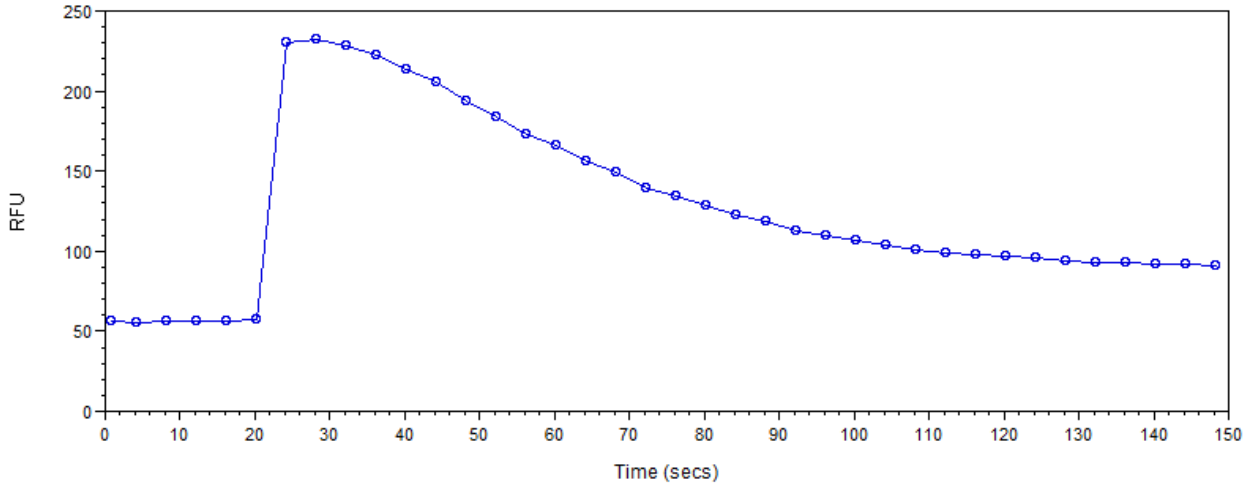


Figure 4.5 Pharmacological inhibition of palmitoylation dose-dependently inhibits TP-mediated calcium mobilization.

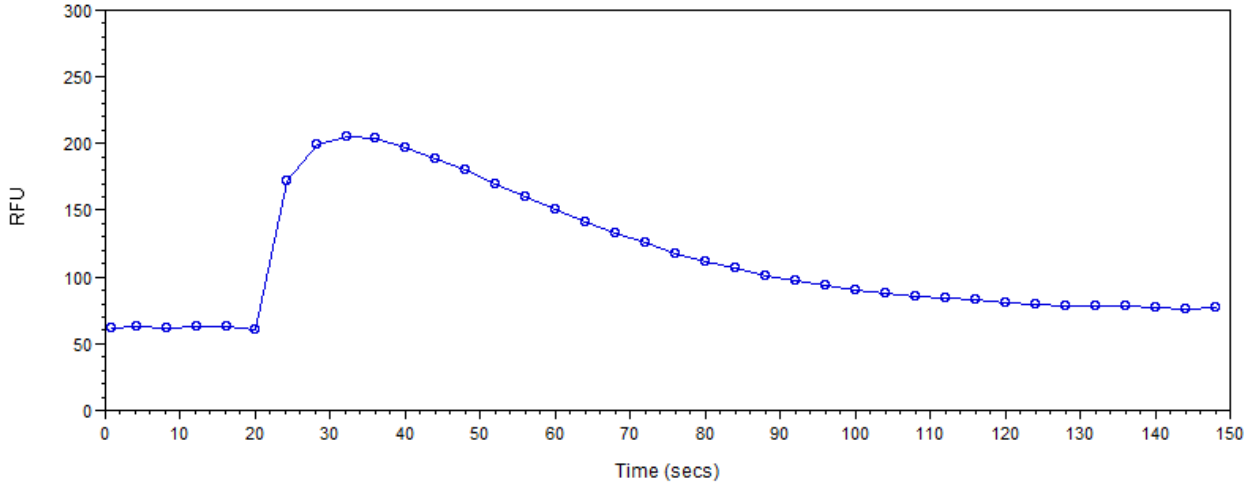
HEK293T cells stably expressing human TPα were cultured in hypoxia (10% O2) or normoxia (21% O2) for 72 hours and incubated with 2-BP (10, 50, or 100 μM) for 4 hours, loaded with calciometric dye Fluo 4NW, and challenged with TP agonist U46619. Agonist-induced Ca^{2+} mobilization was measured in real time and is expressed as relative fluorescence units (RFU) after baseline subtraction (n = 3 separate experiments). †log EC_{50} and max Ca^{2+} mobilization significantly different from normoxic control.*logEC_{50} and max Ca^{2+} mobilization significantly different from hypoxic control (P< 0.01).

A



Well A4
Max - Min 176.55

B



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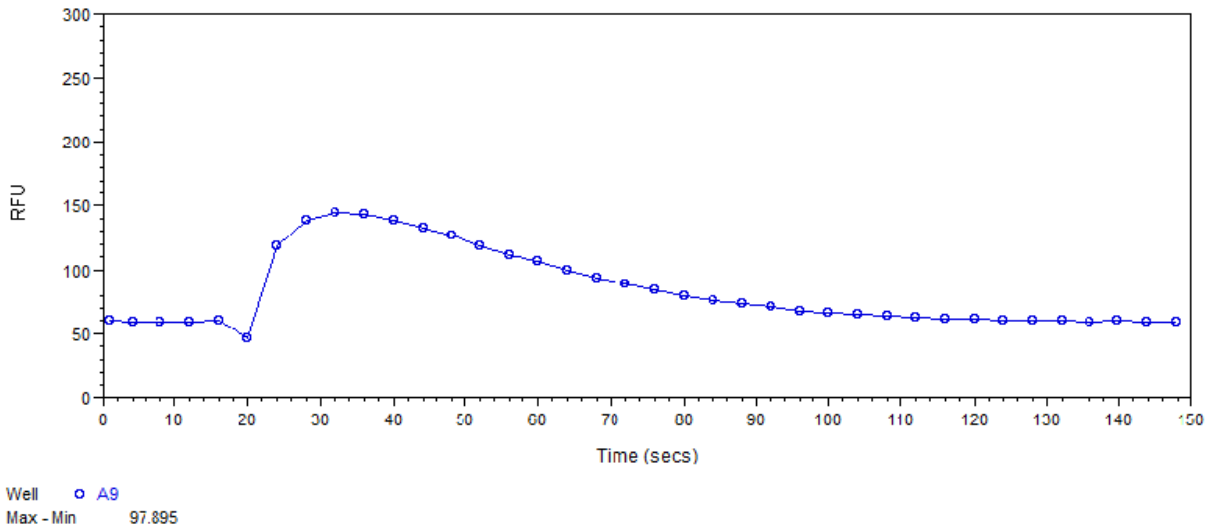
C



D

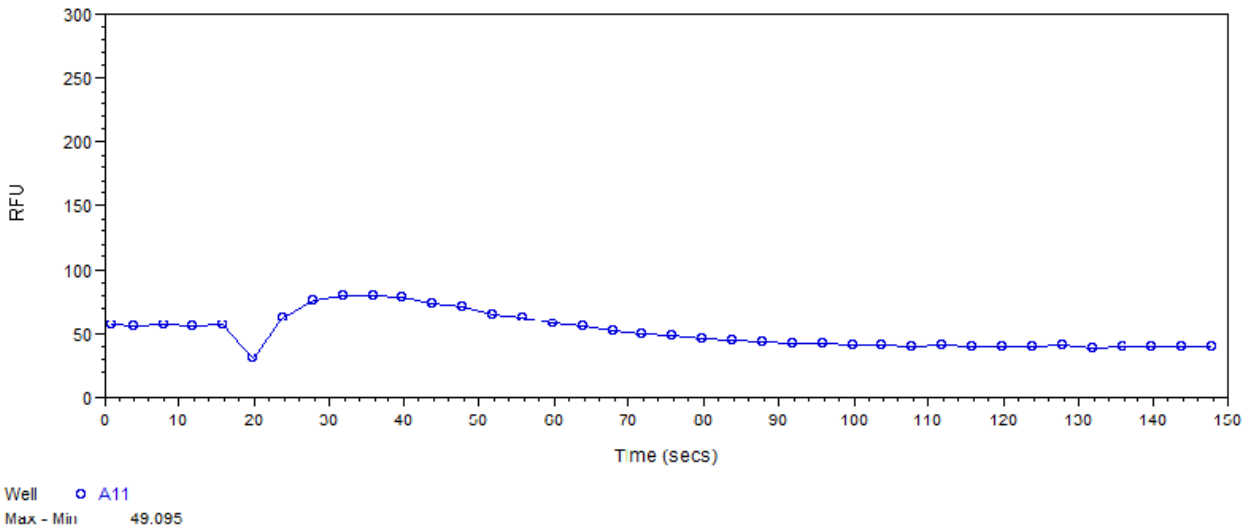


Figure 4.6 Representative calcium traces showing the effect of various doses of 2-BP on U46619-stimulated calcium mobilization

Normoxic conditions are shown. **A** (untreated), **B** (2-BP, 10μM), **C** (2-BP, 50μM), **D** (2-BP, 100μM).

4.1.6 Effect of cysteine substitutions on Gαq localization and TP-mediated calcium mobilization

Western blot of HEK293T whole cell lysates showed equal expression of wild-type Gαq and single (C9A, C10A) and double C9A/C10A mutants **(Figure 4.7A)**. TPα coimmunoprecipitated with wild-type Gαq and with single cysteine substitution Gαq mutants, whereas association of TPα with the palmitoylation-null double mutant C9A/C10A was markedly decreased compared with WT-Gαq **(Figure 4.7B)**. Blotting of eluted proteins from beads absent immune sera (precleared lysates) resulted in no visualized bands. Wild-type Gαq was localized to the membrane fraction. Mutation of one or both palmitoylatable cysteines significantly decreased membrane localization **(Figure 4.7C).** Single cysteine to alanine substitution mutant caused 30 percent whereas double substitution mutant C9A/C10A caused 80 percent drop in TP-stimulated calcium mobilization suggesting that Gαq cysteine mutations had a dose-dependent effect on TP-induced calcium response **(Figure 4.7D)**.

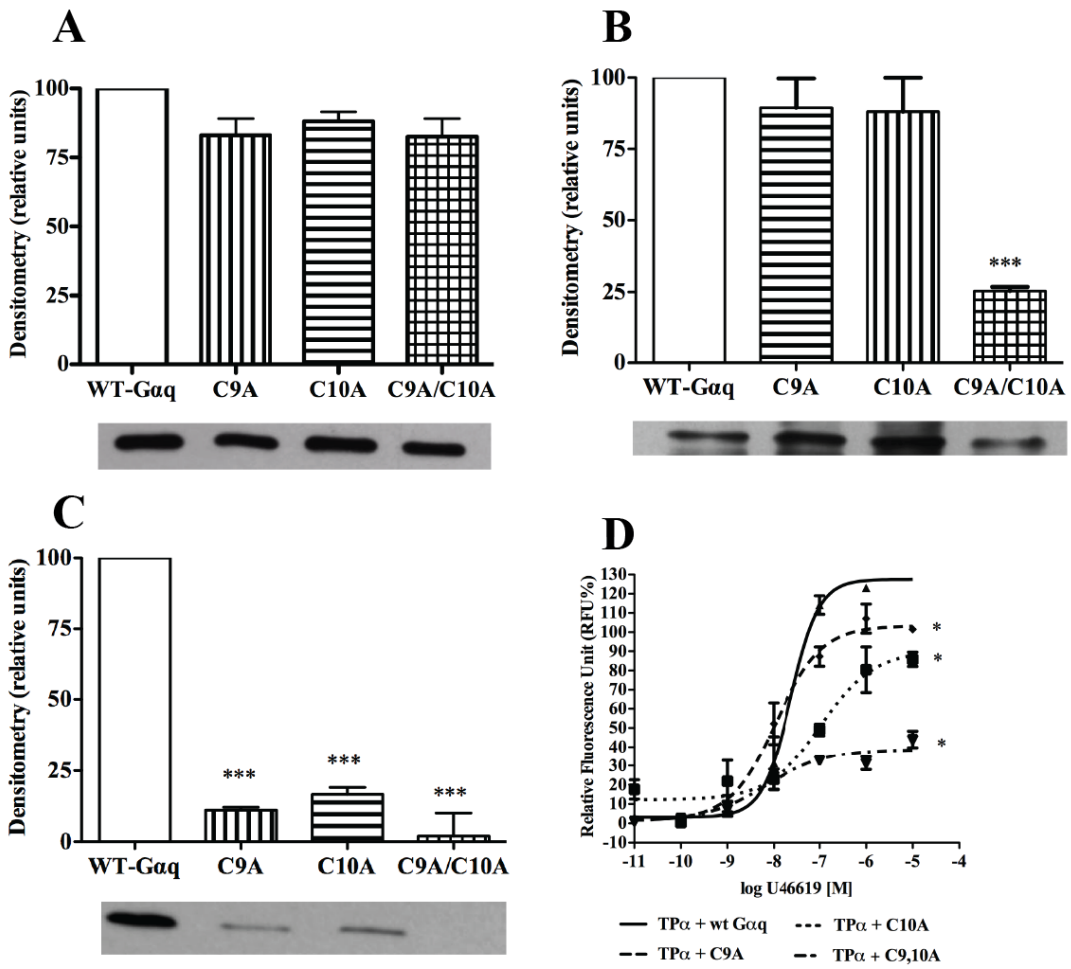


Figure 4.7 Effect of Gαq cysteine substitutions on Gαq localization and on TP-mediated calcium mobilization.

(A) Whole cell lysates from HEK293T cells stably expressing TPα and transiently transfected with wild type EE-tagged Gαq or Gαq single (C9A and C10A) and double (C9A/C10A) cysteine-to alanine mutants were obtained 72 hours after transfection, separated by SDS-PAGE, and probed with anti-EE antibody. Chemiluminescence was normalized to wild-type Gαq. **(B)** TPα was immunoprecipitated using anti-Rho1D4 antibody from whole lysates, and blots were probed for wild-type Gαq and Gαq single and double cysteine mutants using antibody to the EE tag. IB, immunoblot; ***different from wild type ($P < 0.001$). **(C)** Membrane proteins were fractionated by differential centrifugation, separated by SDS-PAGE, and probed with anti-EE antibody. IB, immunoblot; ***different from wild type ($P < 0.001$). **(D)** The role of palmitoylable Gαq cysteines for TP-mediated Ca^{2+} mobilization in HEK293T cells stably expressing human TPα and transiently transfected with wild-type Gαq or single and double mutants was determined upon stimulation with logarithmic dose increments of TP agonist U46619. Agonist-induced Ca^{2+} response was measured 24 hours after transfection by Fluo 4NW (n = 3 separate experiments). *log EC_{50} and max Ca^{2+} different from TP-wt Gαq ($P < 0.05$).

4.1.7 Mutation of Gαq palmitoylable cysteines decreases calcium mobilization upon TP stimulation

In HEK293T cells stably expressing wild type TPα and transfected with wild-type Gαq, 72 hours of hypoxia nearly doubled the calcium response to TP agonist U46619. Cysteine-to-alanine single mutants C9A and C10A decreased U46619-induced Ca^{2+} mobilization to a level equal to normoxic wild-type Gαq. Agonist challenge of TP with C9A/C10A resulted in a 60% drop in hypoxic calcium mobilization **(Figure 4.8)**.

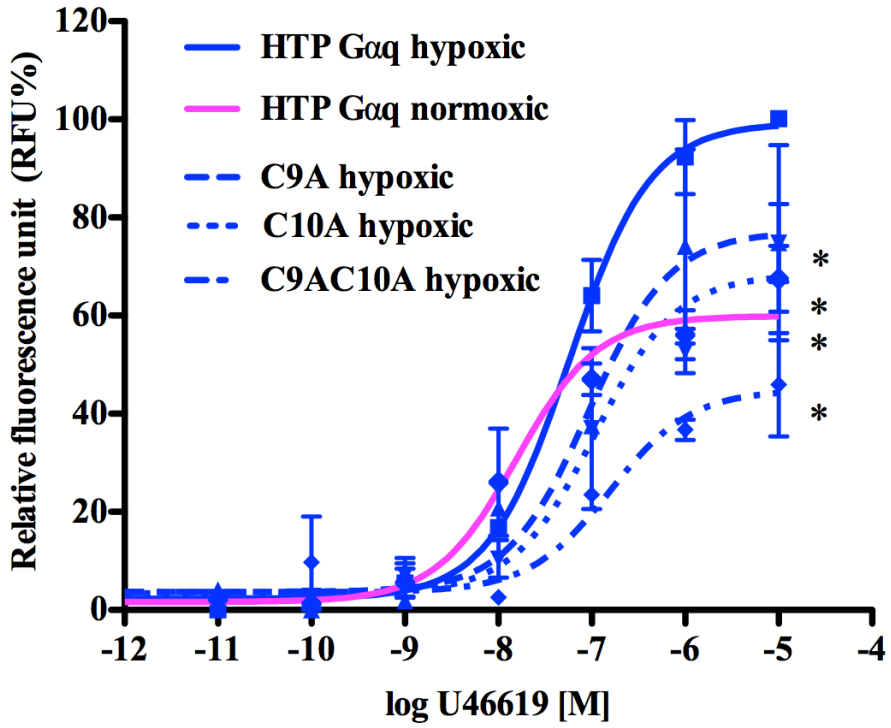


Figure 4.8 Effect of Gαq cysteine substitutions on TP-mediated calcium mobilization under hypoxic conditions.

HEK293T cells stably expressing human TPα and transiently transfected with wild-type (wt) Gαq or single (C9A and C10A) and double (C9A/C10A) cysteine-to-alanine mutants were exposed to hypoxia for 72 hours after transfection, loaded with calciometric dye Fluo4NW, and challenged with logarithmic dose increments of TP agonist U46619. Agonist-induced Ca^{2+} mobilization was measured in real time and is expressed as RFU after baseline subtraction and normalized to hypoxic untreated control. A normoxic untreated group is included as a reference value (n = 3 separate experiments). *log EC_{50} and max Ca^{2+} different from TP-wt Gαq.

4.2 Discussion (Palmitoylation study)

In this study, we examined, using pharmacological and mutational approaches, the role of Gαq palmitoylation in thromboxane receptor signaling in hypoxic and normoxic porcine pulmonary artery rings, pulmonary artery, and aortic myocytes and the role of palmitoylable cysteine residues of Gαq in HEK293T cells stably expressing TPα under hypoxic and normoxic conditions. Our results led to the following conclusions: (*1*) The inhibition of palmitoylation reduces calcium mobilization and contractile force to thromboxane challenge in hypoxic PPHN but not in normal pulmonary artery; (*2*) these effects of palmitoylation inhibition on thromboxane-induced calcium mobilization are peculiar to the pulmonary artery and are not seen in systemic circulation; (*3*) palmitoylation is required for thromboxane-induced release of second messenger IP_3, focusing attention on the palmitoylation state of the TP-G protein complex; (*4*) hypoxia specifically increases the palmitoylation of Gαq; (*5*) the magnitude of thromboxane-induced calcium response is dose dependent on whole cell palmitoylation state and on the degree of Gαq cysteine palmitoylation; (*6*) palmitoylation of a single Gαq N-terminal cysteine residue is required for its physical association with the TPα receptor, whereas palmitoylation of both cysteines is required for Gαq membrane localization and Ca^{2+} mobilization to agonist challenge; and (*7*) depalmitoylation of any one Gαq cysteine residue reduces the contractile response to thromboxane challenge of hypoxic cells to equal that of normoxic cells. We therefore infer that pulmonary arterial hyperresponsiveness to thromboxane is at least in part mediated by increased palmitoylation of Gαq in the pulmonary circuit, which facilitates the association of Gαq with TP.

There is little literature on the role of Gα subunit palmitoylation in hypertensive disorders or in hypoxic disorders. Because protein-specific palmitoylation is difficult to ascertain *in vivo*, to examine the importance of palmitoylation to the GRPC-mediated vasoconstrictor response after

chronic hypoxia *in vivo*, we started with the pharmacological inhibitor of palmitoyl transferase, 2-BP (IC_{50}, 10 μM) [598]. The use of an irreversible palmitate acyltransferase (PAT) inhibitor permitted observation of time-sensitive biological effects of posttranslational modification in a moderately abundant protein in whole tissues and in cells. The specificity of the effect of hypoxia on Gαq acylation, trafficking, and signaling was independently confirmed by metabolic labeling of hypoxic versus normoxic Gαq with ^{3}H-palmitate and by cysteine substitution (which prevents palmitoylation but alters no other biological activity of Gαq). It is reported that infusion of norepinephrine, an adrenergic vasoconstrictor, decreases palmitoylation of Gαq *in vivo*, resulting in uncoupling of several GPCRs from their cognate Gα proteins and in desensitization of the contractile response in systemic arteries [461]. Phenylephrine also stimulates Gαq palmitoylation in aortic membranes, increasing adrenergic receptor coupling with Gαq [460]. It is possible that hypoxia increases Gαq palmitoylation through vasoconstrictor activity or that it has a direct stimulatory effect on protein-specific acyl transferase activity. We have measured thromboxane generation during *in vitro* hypoxia and saw no increase [179], but it is worth investigating whether TP-Gαq association is promoted indirectly in hypoxia by the action of other agonists. In our study, inhibition of palmitoylation diminishes the thromboxane-mediated contraction of pulmonary hypertensive but not control arteries. We observed this phenomenon of 2-BP–sensitive TP agonist hyperresponsiveness after *in vitro* hypoxia in pulmonary artery but not in aortic smooth muscle, indicating that the effect of hypoxia may be specific to the pulmonary circuit.

TPα is a rarity in that it is not amenable to palmitoylation; the majority of GPCRs, including TPβ, can be palmitoylated. To determine whether depalmitoyation impairs signaling of other nonacylated receptors, we challenged nonpalmitoylatable pulmonary arterial purinergic

(predominantly P2Y) receptors with ATP. P2Y receptors are GPCRs that couple with Gαq to mobilize calcium [599], but they also cause pulmonary vasodilation by an unknown switch in Gα coupling selectivity [600]. We observed no difference in calcium response to ATP between normoxia and hypoxia (we have previously reported this pathway in detail [159]. 2-BP modestly decreases ATP-induced calcium responses of hypoxic pulmonary myocytes alone.

TP receptor expression extends distally into the pulmonary vascular circuit in hypoxic pulmonary hypertension. Hypoxia does not alter smooth muscle TP content; cell surface TP localization decreases owing to agonist-induced receptor internalization [161]. Hypoxia decreases TP phosphorylation, resulting in agonist hypersensitivity despite unchanged thromboxane production [159, 179, 532]. TP mobilizes calcium by multiple mechanisms, including opening of L-type calcium channels, calcium-induced calcium release, and endoplasmic calcium release from IP_3–gated channels after Gαq activation. We demonstrate that palmitoylation inhibition ablates IP_3 generation, indicating that palmitoylation specifically alters Gαq activity. Although hypoxia does not alter whole cell palmitate uptake relative to new protein synthesis, it disproportionately increases Gαq palmitoylation, which may facilitate its coupling to vasoconstrictor GPCRs and thereby hyperreactivity of hypoxic Gαq-coupled receptors. This is a novel finding.

For certain receptors, including TPβ, endothelin, and β2-adrenoceptor, C-terminal palmitoylation is required to signal though G-proteins [518, 601, 602] or to selectively switch between Gα subunits [603]. On the other hand, several studies have indicated that N-terminal palmitoylation of Gα subunits is required for their interaction with GPCRs. Palmitoylation ensures membrane localization of Gαs and facilitates its upstream interactions with β-adrenergic receptor and

downstream with adenylyl cyclase [604]. Although the palmitoylation-resistant α1b-adrenoceptor can couple to Gα11, depalmitoylated Gα11 cannot couple the receptor [567]. G protein α subunits typically undergo S-palmitoylation at their amino terminus via reversible post-translational enzymatic addition of a 16-carbon palmitoyl group [419]. Palmitoylation, determined by the activity balance of PAT and acylthioesterase enzymes, mediates protein scaffolding and membrane interactions and thus controls a protein's biological activity [605]. The hydrophobic palmitoylated region can provide a secondary stable membrane tether for proteins that have already been targeted to the membrane compartment due to weak or transient protein–protein interactions via prenylation or myristoylation [606].

Wedegaertner and colleagues [455] reported that both Gαq cysteines need to be palmitoylated for effective membrane localization. Although one palmitoylated cysteine was sufficient for IP_3 production, they found a 50% decrease in IP_3 production due to α2AR stimulation with Gαq cysteine-to-serine single mutants. Our data on Gαq substitution mutations are consistent in that palmitoylation is required for Gαq membrane attachment; very low levels of single cysteine Gαq mutants were localized to the membrane, and C9A/C10A was not found in the membrane fraction. This confirms that palmitoylation plays a crucial role in G-protein trafficking to the cell membrane. There are differing reports regarding localization of nonpalmitoylatable Gαq mutants; Edgerton and colleagues [458] reported in COS cells that C9A/C10A could be pelletted in membrane fractions, whereas others found, as we did, that C9A/C10A was not pelletable [455]. We have previously reported coimmunoprecipitation of native TP with various Gα species to determine the selective coupling of resting or activated TP receptor with its candidate G proteins [565]. In the present study, coimmunoprecipitation of TPα with single and double Gαq mutants reveals varying degrees of GPCR–G-protein interaction, depending on the

palmitoylation capability of Gαq. Although the single cysteine mutants could still bind TPα, a drastic decrease in association of Gα protein with TPα was observed with the unpalmitoylatable Gαq double mutant.

Thus, we speculate that palmitoylation sites are not only important for G protein trafficking to the membranes but may provide initial hinge binding sites for the G protein to the receptor. Our Ca^{2+} mobilization studies fill in the detail that single cysteine-to-alanine Gαq mutants decrease TPα-mediated Ca^{2+} mobilization by 30% and double mutant C9A/C10A by 80%; in other words, the Ca^{2+} response is dose dependent upon Gαq palmitoylation. The sharp decline in membrane localization of single cysteine mutants gives rise to lesser declines in TP-mediated calcium mobilization. We speculate that decreased membrane association of non–receptor-associated Gα subunits, in the presence of a large TPα receptor reserve, could explain the disproportion in the effector–response relationship between loss of Gαq palmitoylation and loss of Ca^{2+} signal [607].

In the absence of membrane localization of TP-Gαq, other TP-associated Ca^{2+} mobilization pathways may account for residual agonist-induced Ca^{2+} signal. The response of TPα coexpressed with wild-type Gαq was almost doubled under hypoxic conditions; Gαq single cysteine substitution decreased the hypoxic response to normoxic levels, indicating that palmitoylation is important for the hyperresponsiveness of hypoxic TP. The double cysteine mutant still generates a modest calcium response under hypoxic conditions but one that is far lower than that of the normoxic wild-type Gαq.

Several studies associate palmitate turnover with post agonist receptor-mediated activation or deactivation of Gαs [437] and Gαq [462] subunits, such that G protein depalmitoylation occurs upon GPCR activation and repalmitoylation coincides with deactivation. Dissociated Gα may be

a better substrate for acylthioesterase [425]. In the current study, we have not examined the effect of ligand binding to TP receptor on palmitate turnover but focused on the effect of resting Gαq palmitoylation on the initiation of receptor-mediated activity. For TP receptors, signaling hyperreactivity of the activated receptor is mediated by increased G-protein activity and thereby increased IP_3 generation, whereas the alacrity of post ligand receptor internalization has little effect on TP agonist responses [161]. We therefore considered post ligand palmitoylation of Gαq not directly relevant to this study.

Protein palmitoylation results from the action of any of 23 DHHC-motif PAT isoforms, which are tightly regulated by substrate sequence and localization. Substrate targets have been identified for certain PATs [608, 609]; PAT 3 and 7 are postulated to palmitoylate Gαq (13). We speculate that hypoxia may increase activity of the PAT relevant to pulmonary arterial Gαq. There are two classes of human PAT isoforms: type I PATs, targeting farnesylated C-terminal cysteines found in oncogenes including H-, N-, and K2A-Ras, and type II PATs that target N-terminal myristoyl-dependent palmitoylation motifs. PAT type I– and type II–selective competitive inhibitors have been studied using high-throughput screening [610]. This holds promise for selective therapeutic targeting of Gαq palmitoylation in PPHN.

Our data cumulatively indicate that hypoxia increases palmitoylation of Gαq, thus facilitating the association of Gαq with TP and increasing TPα-mediated vasoconstrictor responses in the pulmonary circuit. To our knowledge, this is the first report using pharmacological and mutational approaches and a full dose–response study of calcium mobilization to investigate the role of Gαq palmitoylation in GPCR signaling via a nonpalmitoylated vasoconstrictor receptor in the context of vascular hypertension or hypoxia. Our findings on the role of palmitoylation

regulating Gαq-TPα interactions may be generalized to understanding the signaling of other Gαq-coupled vasoconstrictor receptors regardless of the palmitoylation state of the receptor itself. The effect of hypoxia on the flux of protein palmitate turnover merits further investigation.

4.3 Results (TP phosphorylation study)

4.3.1 Effect of *in vivo* hypoxia on pulmonary artery TP responses

Pulmonary arteries from PPHN animals were sensitized to TP stimulation, resulting in a hyper-reactive force response to thromboxane challenge, compared with newborn or age-matched controls **(Figure 4.9A).** TP-stimulated pulmonary artery contractile force decreases during first three days of life in piglets. Myocytes from hypoxic PPHN pulmonary arteries exhibited diminished TP serine phosphorylation, even following primary culture under normoxic conditions **(Figure 4.9B).**

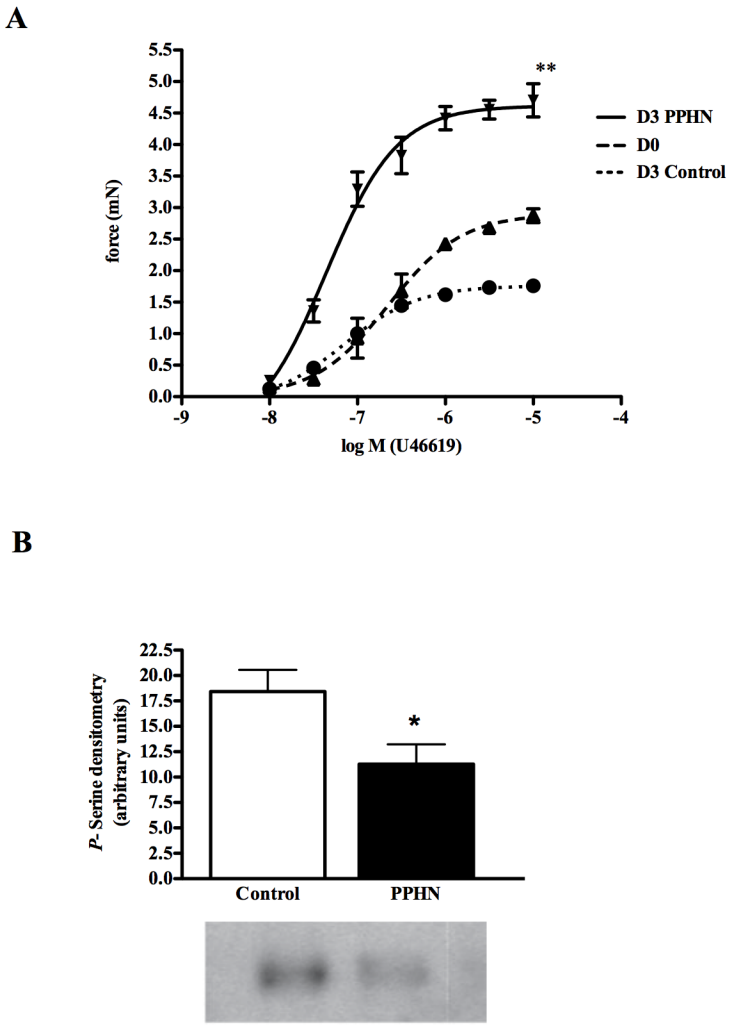


Figure 4.9 TP receptor phosphorylation is decreased in pulmonary artery myocytes from PPHN animals.

(A) Pulmonary arterial rings from newborn (D0), 3-day-old normoxic (D3 Control) and hypoxic pulmonary hypertensive animals (D3 PPHN) were subjected to isometric myography (set at optimal length by length–tension curve; force measurements normalized to maximal KCl-induced force). n = 4 animals per group. Hypoxic PPHN increases TP receptor-mediated contraction, **P < 0.001. **(B)** Representative blot and histogram showing phosphorylation of TP receptors in contractile pulmonary arterial myocytes primary cultured from 3 day hypoxic PPHN swine and age-matched normoxic controls. Cell lysates were subjected to immunoprecipitation using polyclonal antibody (rabbit) to thromboxane receptor, and TP serine phosphorylation quantified in immunoprecipitates by Western blot (mouse monoclonal antibody to phospho-serine; *P < 0.05, n = 8).

4.3.2 Effect of *in vitro* hypoxia on TP receptor-specific GTPase activity

TP receptor-specific membrane GTPase activity was measured as inorganic phosphate release over time (after subtracting basal Pi release), indicative of cumulative GTP hydrolysis during the period of stimulation rather than the rate of hydrolysis. Increased GTP hydrolysis in hypoxic pulmonary artery myocytes infers greater cumulative G protein activation by the TP receptor **(Figure 4.10).** Treatment of membranes with SQ29548 prior to TP challenge abolished the difference in GTPase activities between hypoxic and normoxic groups.

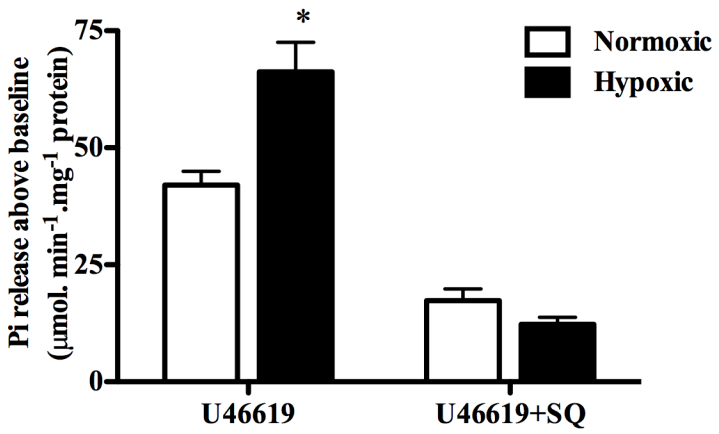


Figure 4.10 TP receptor-specific GTPase activity is increased in hypoxic pulmonary artery myocytes.

Neonatal pulmonary artery myocytes grown in hypoxia or normoxia, challenged for 2 min with TP receptor agonist 10^{-6} M U46619, with or without TP receptor blockade by 10^{-5} M SQ29546, then flash-frozen. GTPase activity quantified in membrane fractions. Organic Pi liberated by membrane GTPase from substrate GTP in response to U46619 was calculated from a standard curve as [Pi] $min^{-1} \cdot mg^{-1}$ protein, after subtraction of baseline (unstimulated) Pi release. Measured GTPase activity represents Pi release specifically due to TP-G-protein complex GTPase activity. This activity is sensitive to TP receptor blockade (+SQ) (data from three separate experiments in triplicate). *$P < 0.05$.

4.3.3 Effect of *in vitro* hypoxia on protein kinase and TP receptor activities in HEK293T cells

After 72 h of in vitro hypoxia, porcine and human TP isoforms demonstrated hyper-responsiveness resulting in increased agonist induced Ca^{2+} mobilization compared to normoxic conditions **(Figure 4.11 A, B).** Hypoxia reduced PKA activity in HEK293T cells and this reduced PKA activity was restored to normoxic levels by treatment with an AC activator, forskolin, or with PDE-3 inhibitor, milrinone. Inhibition with H8 decreased the normoxic PKA activity to the hypoxic level **(Figure 4.12A).** Correspondingly, inhibition of PKA increased TP receptor-mediated Ca^{2+} mobilization in HEK293T cells stably transfected with wild-type TP; PKA activation reduced TP Ca^{2+} mobilization **(Figure 4.12B).** Hypoxia did not significantly reduce PKC activity however hypoxic cells treated with PKC inhibitor Go6983 had significantly lower PKC activity than normoxic treated cells. PKC was effectively activated by PMA in hypoxic and normoxic cells **(Figure 4.12C).** Both the PKC activator and inhibitor reduced the potency of TP receptor-mediated Ca^{2+} mobilization **(Figure 4.12D).**

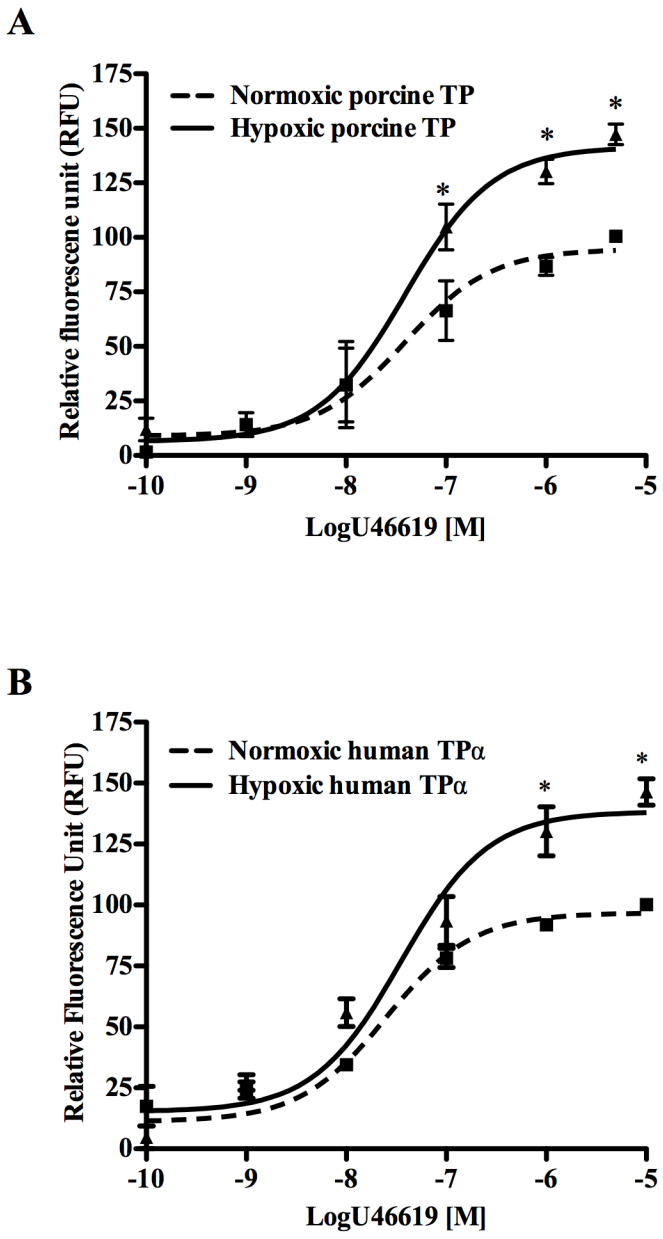


Figure 4.11 Human and porcine TP receptors expressed in HEK293T cells are hyper-responsive after 72 hours of hypoxia.

HEK293T clones stably expressing wild-type porcine TP **(A)** or human TPα **(B)** were exposed to hypoxic or normoxic culture conditions for 72 hours, loaded with calciometric dye fluo-4 and challenged with serial concentrations of the thromboxane mimetic U46619. Ca^{2+} mobilization response to U46619 challenge under normoxic or hypoxic conditions quantified as relative fluorescence units following baseline subtraction; *$P < 0.001$, $n = 4$.

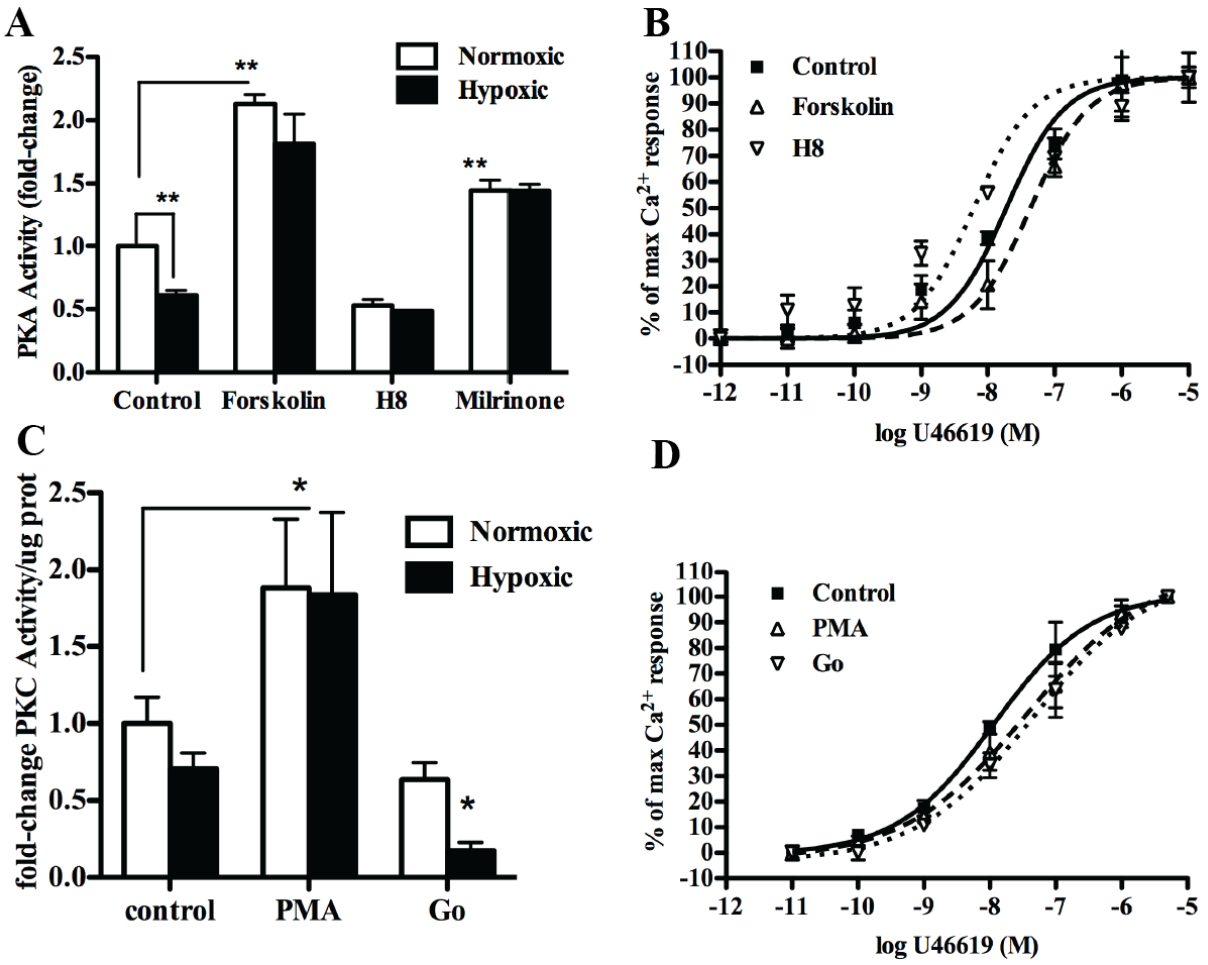


Figure 4.12 PKA, but not PKC, activity in HEK 293T cells is reduced by hypoxia.

After 72 h of hypoxic or normoxic conditions, HEK293T cells were incubated with 10^{-6} M forskolin (activates PKA), 10^{-6} M H8 (inhibits PKA), 10^{-8} M milrinone (PDE-3 inhibitor), PMA (activates PKC), 10^{-6} M Go6983 (inhibits PKC) or diluent. PKA **(A)** or PKC **(C)** activity measured in cell lysates, expressed as fold-change from normoxic control. TP-mediated Ca^{2+} mobilization in the presence of PKA activation or inhibition **(B)** or PKC activation or inhibition **(D)** determined using fluo-4, quantified as relative fluorescence units after baseline subtraction, normalized to maximal Ca^{2+} response. n = 4, **P < 0.001 compared with normoxic control.

4.3.4 Expression and ligand binding properties of TP receptor substitution mutants

Based on the NetPhos sequence analysis, residues Ser239 (located on intracellular loop 3) and Ser324, Ser329 and Ser331 (on the C-terminal tail; **Supplemental Figure 7.4**) of the porcine TP and TPα receptor were predicted for phosphorylation with high probability scores (0.994, 0.985, 0.991 and 0.927 respectively). TP receptor saturation binding kinetics, maximal receptor binding (B_{max}) and dissociation constant (K_d, in nM) were determined using cell membrane fractions expressing wild type human TPα, porcine TP or TP receptor substitution mutants, using the TP antagonist [^{3}H]-SQ29548. Among the serine substitution mutants **(Table 4.1)**, Ser239A and Ser329A had K_d values comparable to the wild-type TP receptor. The K_d of Ser324A was lower ($P < 0.05$) than that of the wild-type TP receptor, indicating increased radioligand affinity. The Ser331A K_d was increased ($P < 0.05$) compared with the wild-type receptor. There were no measurable differences in B_{max} for any mutant capable of binding SQ29548.

4.3.5 Effects of PKA and *in vitro* hypoxia on TP responses

Agonist dose–response curves for transiently expressed wild type TPα, Ser239A and Ser329A were similar. Treatment with 10^{-6} M forskolin did not alter agonist dose–response relationships of Ser239A or Ser329A, indicating that these receptors remained maximally phosphorylated, whereas 10^{-6} M H8 treatment sensitized both dose–response curves, indicating that these constructs were amenable to dephosphorylation **(Figure 4.13 A, C)**. Ser324A exhibited a left-shifted agonist response compared with wild type TP receptors. Neither forskolin nor H8 was capable of rectifying the Ser324A Ca^{2+} response to U46619; it remained elevated compared with wild-type TP **(Figure 4.13B)**. The Ser331A TP receptor mutant was relatively desensitized to agonist stimulation compared with wild-type TP receptors. Neither forskolin nor H8 treatment

altered the response of Ser331A to U46619; it remained desensitized in comparison to wild-type TP receptors **(Figure 4.13D)**. A comparison of log EC50 values is given in **Table 4.2**. A heterologous competitive displacement assay demonstrated that for wild-type TP receptors, U46619 displaced labelled antagonist more easily in hypoxia than in normoxia ($P < 0.01$, **Figure 4.14A**). Ser324A showed high affinity binding to U46619 in both normoxia and hypoxia **(Figure 4.14A).** Ser329A bound U46619 with an affinity comparable with that of the native receptor under hypoxic and normoxic conditions **(Figure 4.14B).** Both hypoxic and normoxic Ser331A had affinities for U46619 comparable to that of normoxic wild-type TP receptors **(Figure 4.14C).** A comparison of log K_i values is included in **Table 4.1**. Finally, we examined the effects of C-terminal serine substitution mutants on the hypoxia-induced sensitization of the TP receptor-agonist interaction. Due to loss of transiently transfected cells after 72 h hypoxia exposures, these dose-response curves were obtained after 48 h of hypoxia. Hypoxia still caused relative TP receptor hyper-responsiveness at this time point, although less marked than after 72 h of exposure. Ser324A mutants had a sensitized TP agonist dose-response relationship compared to normoxic cells, which resembled that of hypoxic cells **(Figure 4.15A)**, although without a discernible effect on maximal reactivity. Ser329A had a normal dose-response relationship with no increased sensitivity or changes in maximal calcium mobilization in either hypoxic or normoxic cells **(Figure 4.15B).** Ser331A had a right-shifted (desensitized) dose-response curve and depressed maximal reactivity in both hypoxic and normoxic cells **(Figure 4.15C)**. A comparison of log EC_{50} values is presented in **Table 4.3**.

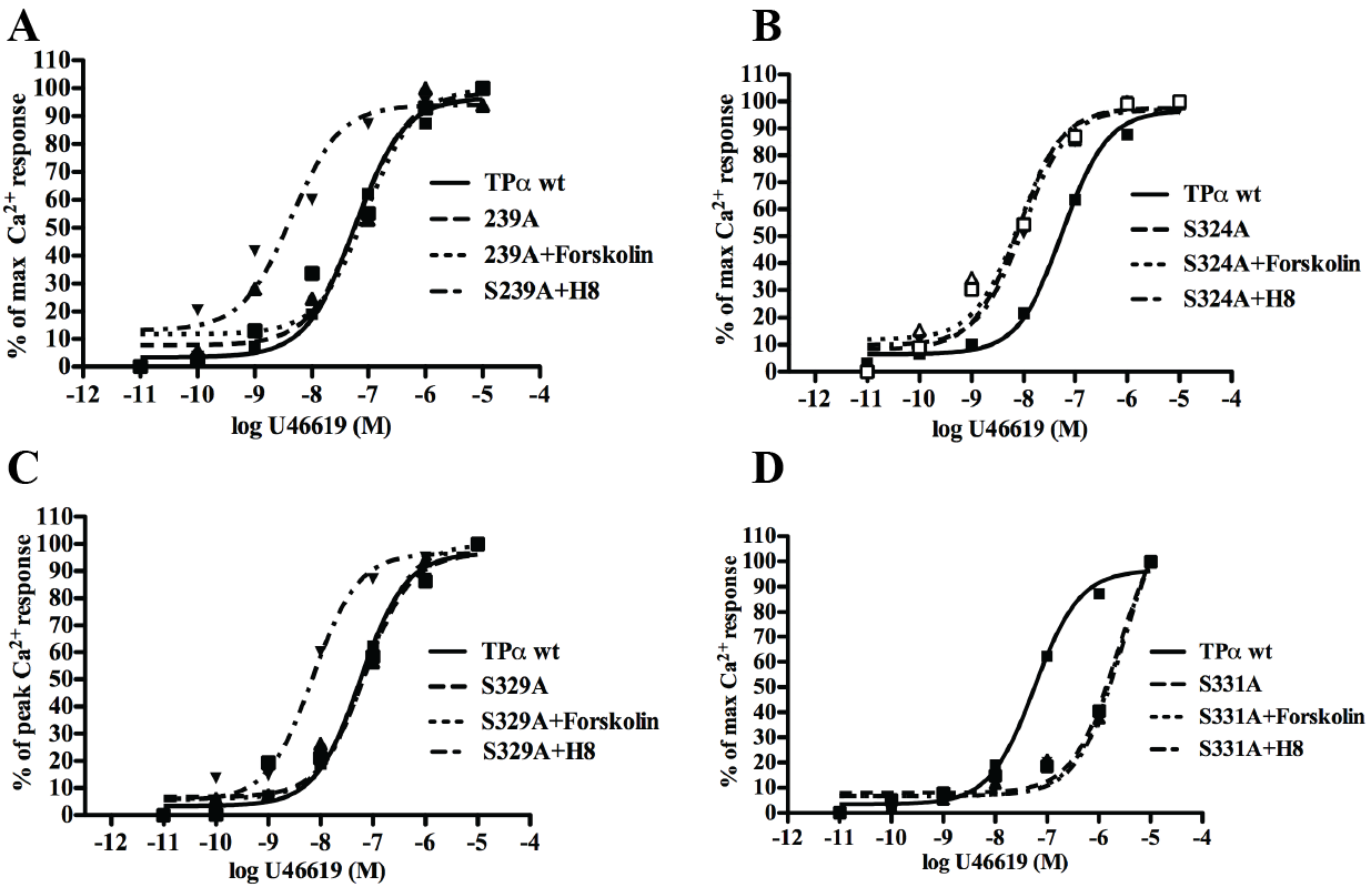


Figure 4.13 PKA sensitivity of wild-type TPα and amino acid substitution mutants.

HEK293T cells transfected with human wild-type TPα, or serine to alanine substitution mutants Ser239A, Ser324A, Ser329A or Ser331A were incubated with 10^{-6} M forskolin (activates PKA), 10^{-6}M H8 (inhibits PKA) or diluent, then challenged with serial concentrations of TP agonist U46619. **(A)** Alanine substitution of Ser239 did not alter PKA sensitivity of the TP agonist concentration-response curve relative to the wild-type TP receptor. **(B)** Substitution of Ser324 sensitized the receptor to the agonist and abolished PKA responsiveness. **(C)** The Ser329A mutant had PKA sensitivity comparable to wild-type TP receptors. **(D)** Ser331 substitution reduced the sensitivity of the receptor to the TP receptor agonist, regardless of PKA activation state. Corresponding EC_{50} values are given in Table 4.2.

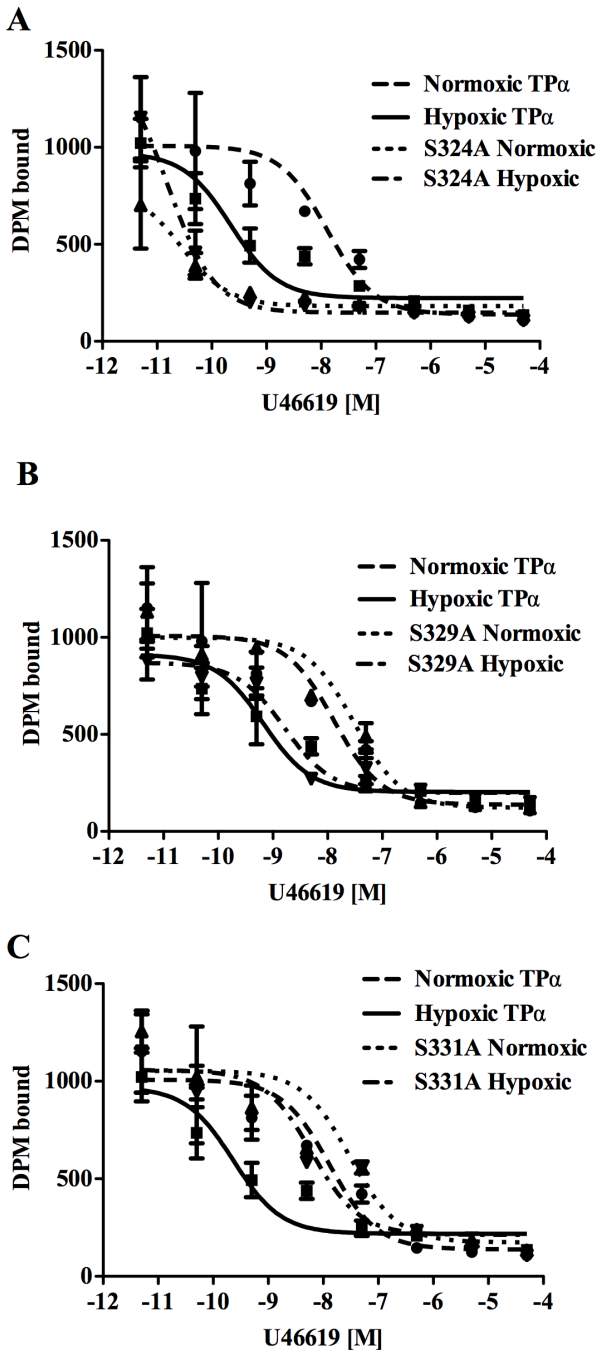


Figure 4.14 Hypoxia increases competitive binding of agonist to TPα; effect of C-terminal amino acid substitution mutation.

HEK293T cells transfected with human wild-type TPα, or serine-to-alanine mutants **(A)** Ser324A, **(B)** Ser329A or **(C)** Ser331A, following 48 h of hypoxic or normoxic exposure, were incubated with a saturating concentration of [^{3}H]-SQ29548, before antagonist displacement with serial concentrations of cold agonist U46619. Data are from three experiments.

Table 4.1 Radioligand binding and displacement kinetics of human TPα and human TP amino acid substitution mutants

Receptor	K_d (nM)[a]	95% Confidence interval	B_{max}[b] pM.mg^{-1}	Normoxic log K_i [c]	Hypoxic log K_i [c]
TPα wt	3.79	2.80 – 4.78	783 ± 119	–8.759	**–10.51**
Ser239A	3.90	3.47 – 4.33	969 ± 48	–	–
Ser324A	**2.23**	1.81 – 2.65	1005 ± 61	**–11.43**	**–11.69**
Ser329A	3.80	2.21 – 5.08	968 ± 45	–8.387	**–9.662**
Ser331A	**5.80**	4.81 – 6.79	857 ± 41	–8.389	–9.082

Radioligand saturation binding kinetics assayed for human wild-type TPα and human TPα serine-to-alanine substitution mutants Ser239A, Ser324A, Ser329A and Ser331A, using labelled TP receptor antagonist [^{3}H]-SQ29548; values from three experiments in duplicate.

[a] Dissociation constant (K_d), expressing inverse of affinity of antagonist SQ29548 for the receptor. Boldface denotes receptor antagonist binding affinity differs from wild type ($P < 0.05$).

[b]Maximum binding (B_{max}) of the antagonist SQ29548 for TP receptor, usually expressed as pmol of the receptor mg-1 total membrane protein; mean ± SEM.

[c]Competitive displacement kinetics under conditions of hypoxic and normoxic cell growth; saturating concentration of labelled antagonist [^{3}H]-SQ29548, displaced with serial concentrations of cold agonist U46619. Ki is the equilibrium dissociation constants, calculated from concentration of agonist inhibiting 50% of antagonist binding. Boldface values differ from normoxic wild type K_i ($P < 0.05$).

Table 4.2 Dose-response relationships of TP substitution mutants in presence of PKA activation and inhibition

Receptor	log EC_{50} untreated	log EC_{50} when PKA activated	log EC_{50} when PKA inhibited
TPα wt	−7.24 ± 0.08	–	–
Ser239A	−7.18 ± 0.22	−7.03 ± 0.27	**−8.36** ± 0.30
Ser324A	**−8.10** ± 0.18	**−8.09** ± 0.23	**−8.00** ± 0.19
Ser329A	−7.16 ± 0.19	−7.14 ± 0.13	**−8.15** ± 0.11
Ser331A	**−5.63** ± 0.17	**−5.60** ± 0.20	**−5.54** ± 0.20

[a]log EC50 = log molar concentration of agonist U46619 that produces 50% of maximal peak calcium mobilization response for TP receptors and TP substitution mutant receptors. Boldface values differ from reference value, untreated wild type ($P < 0.05$).

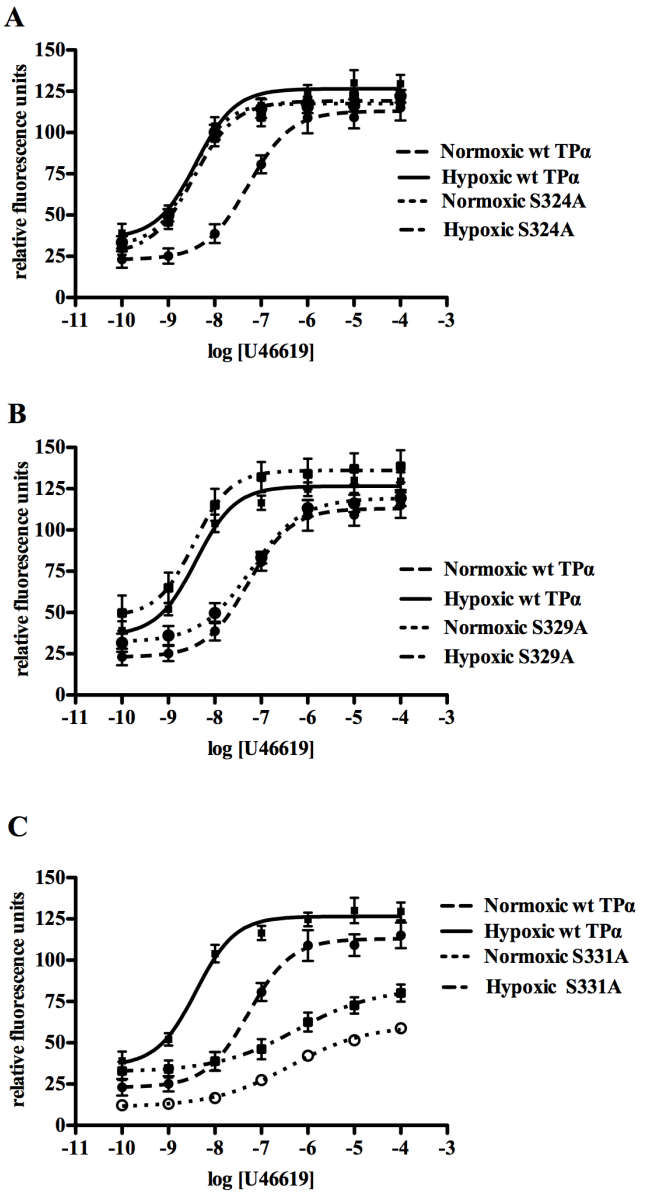


Figure 4.15 Hypoxia increases agonist-induced Ca^{2+} mobilization by TPα; effect of C-terminal amino acid substitution mutation.

HEK293T cells transfected with human wild-type TPα, or serine-to-alanine substitution mutants Ser324A, Ser329A or Ser331A were incubated for 48 h in hypoxic or normoxic conditions, followed by challenge with serial concentrations of agonist U46619. Absolute Ca^{2+} mobilization curves showing **(A)** differing effects of Ser324 substitution on agonist sensitivity in normoxic versus hypoxic conditions, relative to wild-type TP receptors; **(B)** effect of Ser329 substitution on hypoxia sensitivity of TP receptor-mediated Ca^{2+} response; and **(C)** effect of Ser331 substitution on hypoxic and normoxic TP agonist responses.

Table 4.3 Dose-response relationships of TP receptors and TP substitution mutants in normoxia or hypoxia

Receptor	log EC_{50}[a] in normoxia	log EC_{50}[a] in hypoxia
TPα wt	−7.24 ± 0.10	**−8.31** ± 0.09
Ser324A	**−8.42** ± 0.08	**−8.35** ± 0.08
Ser329A	−7.21 ± 0.09	**−8.39** ± 0.15
Ser331A	**−6.44** ± 0.09	**−6.34** ± 0.17

[a]log EC50 = log molar concentration of agonist U46619 that produces 50% of maximal peak calcium mobilization response for TP receptors and TP substitution mutant receptors. Boldface values differ from normoxic wild type ($P < 0.05$).

4.4 Discussion (TP phosphorylation study)

In this study, the phosphorylation potential of TP serine residues was elucidated using *ex vivo* experiments on pulmonary arterial rings, and *in vitro* site-directed mutagenesis of phosphorylation-amenable serine residues.

Hypoxia induces the PPHN phenotype [592, 611] and renders the pulmonary circuit hyper-responsive to thromboxane. Whereas TP receptor isoform switching has been postulated as a mechanism for sensitization, as only one TP receptor isoform is expressed in swine, isoform switching cannot be cited as a rationale for altered signalling in this PPHN model. We have previously shown that *in vitro* hypoxia impairs TP receptor phosphorylation in pulmonary artery myocytes. In this study, we demonstrated for the first time that diminished TP receptor phosphorylation exists in the porcine pulmonary artery after *in vivo* hypoxia, in association with increased force generation to challenge with a thromboxane mimetic. This disease-specific finding provides physiological validation for studying TP receptor phosphorylation *in vitro*.

We used Pi release as an index of GTPase activity following TP receptor agonist challenge, measured using a colorimetric GTPase activity assay kit, in membrane fractions flash-frozen in a 2 minute time window following receptor activation. Pi release measured in membranes from cells unstimulated by TP agonist was measured as background, and was subtracted from agonist-stimulated Pi release. While this serves to exclude GTPase activity unrelated to TP receptor activity, it cannot exclude non-receptor-G protein complex GTPase activity occasioned by the stimulus of TP signaling (for example, due to activation of membrane-associated GTPase enzymes including Rho- or Rac GTPases). These GTPase activities typically peak several minutes following receptor activation, but cannot be excluded within the 2 minute post-

stimulation window as a source of spurious Pi release which would not be eliminated by baseline subtraction. Our data indicate an increase in receptor-complex associated cumulative GTPase activity (denoted by cumulative Pi release) in hypoxic myocytes, which we only interpret as increased total TP receptor complex activity under hypoxic conditions (given the relatively permissive time period of GTPase activity measurement, we did not take this to indicate increased rapidity of signal termination or receptor internalization, although we have published on non-PKA-sensitive hypoxic TP receptor internalization elsewhere [612]. We take these data to bolster our previous finding of increased TP signal in hypoxic myocytes. We then turned to examine mechanisms of PKA-sensitive TP phosphorylation.

The hypoxia and PKA sensitivity of wild-type TP receptors and serine substitution mutants were then assessed to identify specific serine residues responsible for PKA-mediated regulation of TP receptor signalling, which may be important in hypoxic pulmonary hypertension. Multiple regulatory kinases and phosphorylation sites have been studied in relation to TP receptors. The TP receptor is phosphorylated through actions of pulmonary circuit relaxants, particularly PGI_2, resulting in TP receptor desensitization [526, 538]. IP-induced TP phosphorylation has been variously ascribed to actions of PKC [528], G-protein-related kinases [523] or PKA [519, 525, 526]. The major effector of IP receptor-mediated TPα regulation is PKA [613]. PKA plays a role in agonist-induced desensitization of TPα [519] , whereas GRK phosphorylation of Ser239 is implicated in agonist-induced desensitization and internalization of only TPβ [522]. The role of PKC in TP receptor desensitization is unclear. PKC causes an initial reduction in TP receptor-ligand binding followed by a time-dependent increase [614]; inhibition of PKC very modestly increases TP ligand binding [575]. Pharmacological PKC inhibitors cross-react with TP-specific GRKs, necessitating cautious evaluation of TP regulation by PKC [523].

We previously reported decreased serine phosphorylation of TP in pulmonary arterial myocytes after *in vitro* hypoxia and established that loss of PKA- (and not PKC-) mediated TP phosphorylation was a mechanism for thromboxane hyper-reactivity in hypoxia. PKC activation did not decrease TP agonist response [161]. TP agonist sensitivity is tightly regulated by PKA, giving rise to just two characteristic agonist–response relationships corresponding to phosphorylated (desensitized) or dephosphorylated (sensitized) TP receptors [532]. Our current findings confirm that PKA activation and inhibition reciprocally modulate TP receptor-mediated Ca^{2+} release. PKC activation and inhibition do not have this precise effect on the TP dose–response curve. We therefore conclude that PKA-mediated phosphorylation determines TP receptor affinity.

We previously reported that hypoxia impairs basal PKA activity in pulmonary arterial myocytes [532]; in the present study, we confirm this phenomenon in hypoxic HEK293T cells, signalling-intact mesenchymal cells that lack native TP and IP receptors. Stably transfected porcine and human TPα are hyper-responsive to U46619 challenge under hypoxic conditions. Transiently transfected HEK293T cells were less robust under hypoxic conditions, so studies of the transiently transfected TP mutants were carried out after 48 h of hypoxic exposure. Both potency and efficacy of U46619 response are increased after 72 h of hypoxia; in studies carried out after 48 h of hypoxia, there was increased agonist potency but little change in efficacy. All TP mutants studied were expressed with similar B_{max} and were able to bind labelled antagonist. We focused our attention on C-terminal serines, commonly implicated in receptor regulation. Whereas Ser239A and Ser329A had ligand binding parameters similar to wild-type TP receptors, the Ser324A mutation had increased interaction with both agonist and antagonist. Agonist-mobilized Ca^{2+} responses of both the Ser239A and Ser329A mutants exhibited sensitivity to PKA activity,

as did wild-type TP receptors, indicating that these serines are not associated with regulatory phosphorylation of TP receptors by PKA.

Ser324 has been implicated in PKA-mediated regulation of TP receptors [524, 525], although due to differing residue enumeration between research groups, this specific residue is identified in some studies as Ser329. *In vitro* studies have shown that Ser324 is only marginally phosphorylated by PKC [615]. In our hands, Ser324A bound to U46619 with high affinity compared with wild-type TPα. The dose–response relationship for Ca^{2+} mobilization showed that this receptor was hypersensitive to agonist stimulation compared to control TP receptors. Ser324A Ca^{2+} signalling was insensitive to PKA activation or inhibition, and this construct remained sensitized to agonist stimulation under normoxic conditions, indicating its resistance to regulatory phosphorylation. Hence, we concluded that phosphorylation of Ser324 may be responsible for the PKA-mediated down-regulation in the responsiveness of TP receptors to agonists in the normoxic pulmonary artery.

In contrast, the Ser331A TP mutant showed mildly decreased affinity for agonist and antagonist, compared with wild-type TPα. Substitution of this residue renders it insensitive to PKA inhibition and activation, but with a consistently desensitized agonist dose–response curve and a depressed agonist response in both hypoxia and normoxia. These data suggest that Ser331 is important for regulating TP receptor function, but that loss of phosphorylation at this locus decreases receptor signalling. Other groups have identified Ser331 as being amenable to the phosphorylation and desensitization induced by both PKG [531] and PKA [530]. Our data do not support this finding, but paradoxically suggest that dephosphorylation of Ser331 may serve to decrease TP receptor activity, without significantly altering membrane localization. We speculate

that substitution of Ser331 reduces the functionality of the receptor protein. Site-specific receptor phosphorylation can promote positive or negative feedback loops for receptor signalling [616]. PKA-mediated phosphorylation of the β_2-adrenoceptor may determine selective G-protein coupling, distinct from mechanisms of desensitization [617]. It is therefore possible that loss of Ser331 phosphorylation due to alanine substitution induces the TP receptor to dissociate from its cognate G-protein, or inhibits regulatory phosphorylation occurring at other sites, resulting in the observed loss of activity.

We propose that Ser324 and Ser331 are important residues regulating TP receptor function by phosphorylation. The Ser324A and Ser331A mutants shift the U46619 concentration–response curve in opposite directions, implying that phosphorylation at these two sites has opposite effects. Ser324 phosphorylation by PKA desensitizes the TP receptor. Ser331 phosphorylation has a relatively moderate sensitizing effect, whereas phosphorylation-resistant substitution of this residue markedly reduces TP receptor signalling activity. Cumulatively, the net effect of PKA activation on native TP (human and porcine) receptors is one of diminished receptor-agonist interaction and decreased agonist-mediated Ca^{2+} mobilization.

Hypoxia induces a loss of PKA-mediated TP receptor phosphorylation in the neonatal pulmonary circuit; the loss of TP receptor phosphorylation and increase in TP receptor-mediated contractility is demonstrable both *ex vivo* and *in vitro*. Ser324 appears to be the critical residue for this dysregulation. We previously demonstrated that hypoxic-induced TP receptor hyper-responsiveness may be alleviated by treatment with the PDE inhibitor, milrinone; we now confirm that Ser324 phosphorylation by PKA is the probable mechanism for this pharmacological effect.

4.5 Results (Adenylyl cyclase study)

4.5.1 Basal AC activity is decreased in hypoxic PASMC

AC activity was determined as loss of terbium norfloxacin luminescence, when unbound from ATP and bound to an inorganic phosphate donated by action of AC. Environmental hypoxia of 10% O_2 paradoxically increases basal substrate ATP abundance in hPASMC (**Figure 4.16A**). AC product formation as Δ fluorescence intensity/sec/unit protein, in presence of excess substrate 100μM ATP, indicates optimal reaction linearity at 33-66 sec, and plateau after 132 sec reaction time in both hypoxic and normoxic PASMC; product formation is decreased by hypoxia (**Figure 4.16B**). AC specific activity versus substrate curve; hypoxic AC has lower catalytic activity, derived by non-linear curve fit. Maximal velocity of AC enzyme: Hypoxic Vmax=162u/mg/min, Normoxic Vmax= 238u/mg/min (*$p<0.001$). Michaelis Menten catalytic constant: Hypoxic Km=83.4μM, Normoxic Km=86.9μM (**Figure 4.16C**).

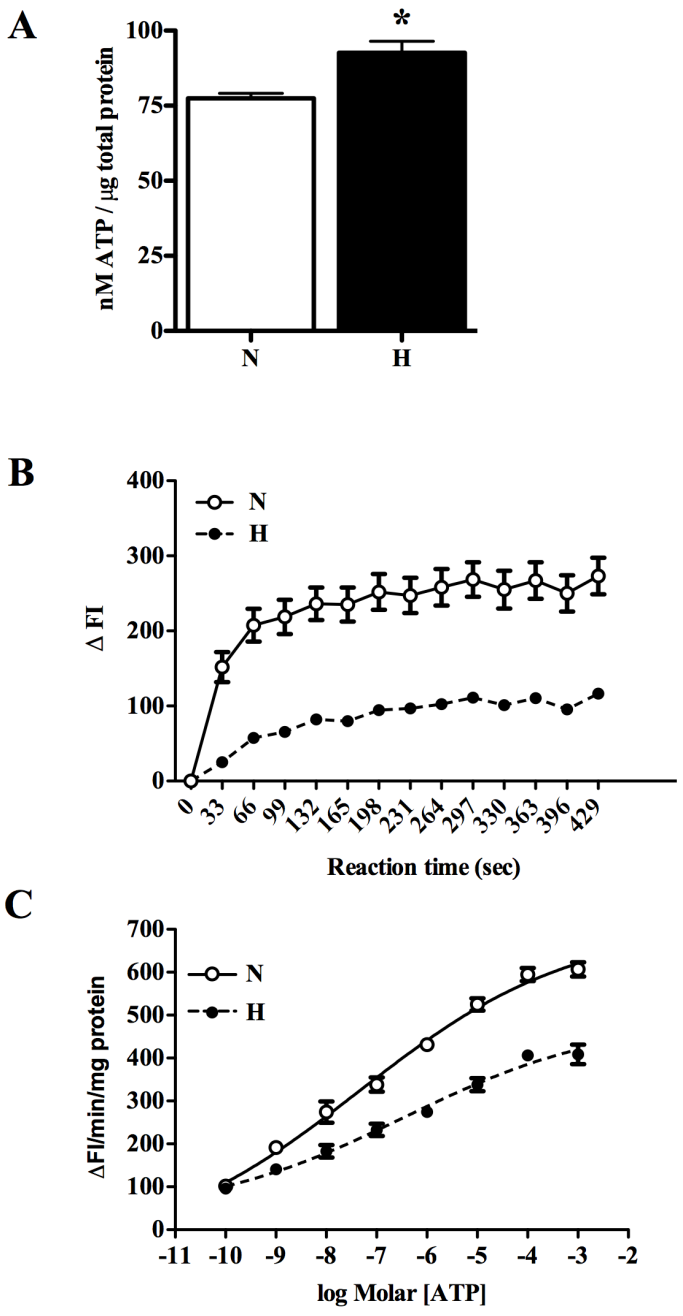


Figure 4.16 Basal adenylyl cyclase activity is decreased in hypoxic PASMC.

(A) Basal ATP abundance in hypoxic (H; 72h 10%O_2, 5%CO_2) compared to normoxic (N; 72h 21%O_2, 5%CO_2) hPASMC (*$p<0.05$). (**B**) AC product formation (ΔFI) over time, in presence of excess substrate (100µM ATP), indicates optimal linearity at 33-66 sec, and plateau after 132 sec reaction time data shown from pPASMC (**C**) AC specific activity versus substrate concentration curve in hypoxic versus normoxic pPASMC.

4.5.2 cAMP-specific PDE activity is decreased in hypoxia

cAMP-specific PDE activity, as Pi release due to cAMP substrate hydrolysis by action of the PDE in lysates, is lower in hypoxic compared to normoxic pPASMC (**Figure 4.17A)**; this decrease is proportional to the decrement in cAMP substrate availability. The cAMP-specific PDE activity in normoxic and hypoxic pPASMC was then separated by selective pharmacological inhibition of PDE1 (vinpocetine), PDE2 (Bay-60-7550), PDE3 (milrinone), or PDE4 (rolipram). Calculation of pharmacologically fractionated cAMP PDE activities as percent inhibition of the basal (uninhibited) total PDE activity revealed that PDE2, 3 and 4 activities account for this difference in measured PDE activity between normoxic and hypoxic myocytes (**Figure 4.17B**).

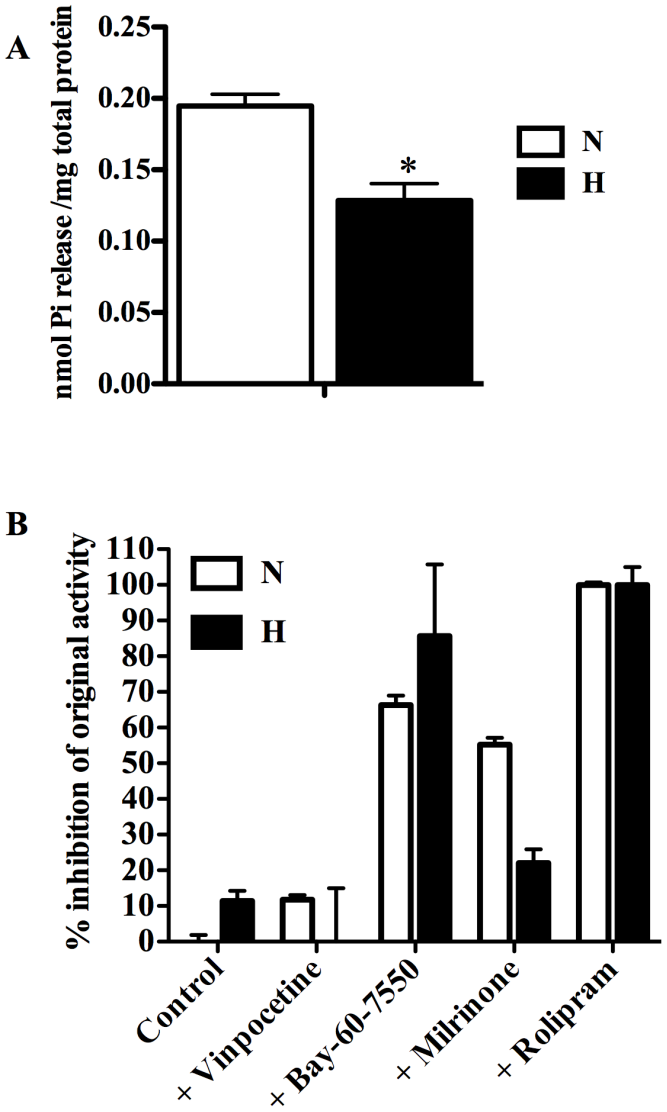


Figure 4.17 cAMP-specific PDE activity is attenuated in hypoxia because of decreased cAMP substrate availability.

(A) PDE activity in hypoxic (H; 72h 10%O_2, 5%CO_2) and normoxic (N; 72h 21%O_2, 5%CO_2) pPASMC (*$p<0.05$). **(B)** Pharmacologically fractionated cAMP-specific PDE activities in N and H, in presence of selective inhibitors of PDE1 (vinpocetine), PDE2 (Bay-60-7550), PDE3 (milrinone), or PDE4 (rolipram), as Pi release due to cAMP substrate hydrolysis by action of PDE in lysates, expressed as percent inhibition of the basal (uninhibited) total PDE activity (N=6; *$p<0.05$ compared to N control; †$p<0.05$ compared to H control).

4.5.3 Resting and stimulated AC activity is decreased in hypoxic PASMC

AC activity after 72 hour hypoxic or normoxic exposure was quantified in porcine (**Figure 4.18A**) and human (**Figure 4.18B**) PASMCs, following prostacyclin receptor stimulation with 1μM iloprost, or receptor-independent activation by direct AC stimulant 1μM forskolin, prior to cell lysis. In all cases, maximal AC activity is significantly higher in normoxic compared to hypoxic myocytes. In both cell types, receptor independent AC activation is impaired in hypoxia; forskolin increases AC activity in hypoxic cells to approach the normoxic baseline, but not to the level achieved in forskolin-treated normoxic myocytes.

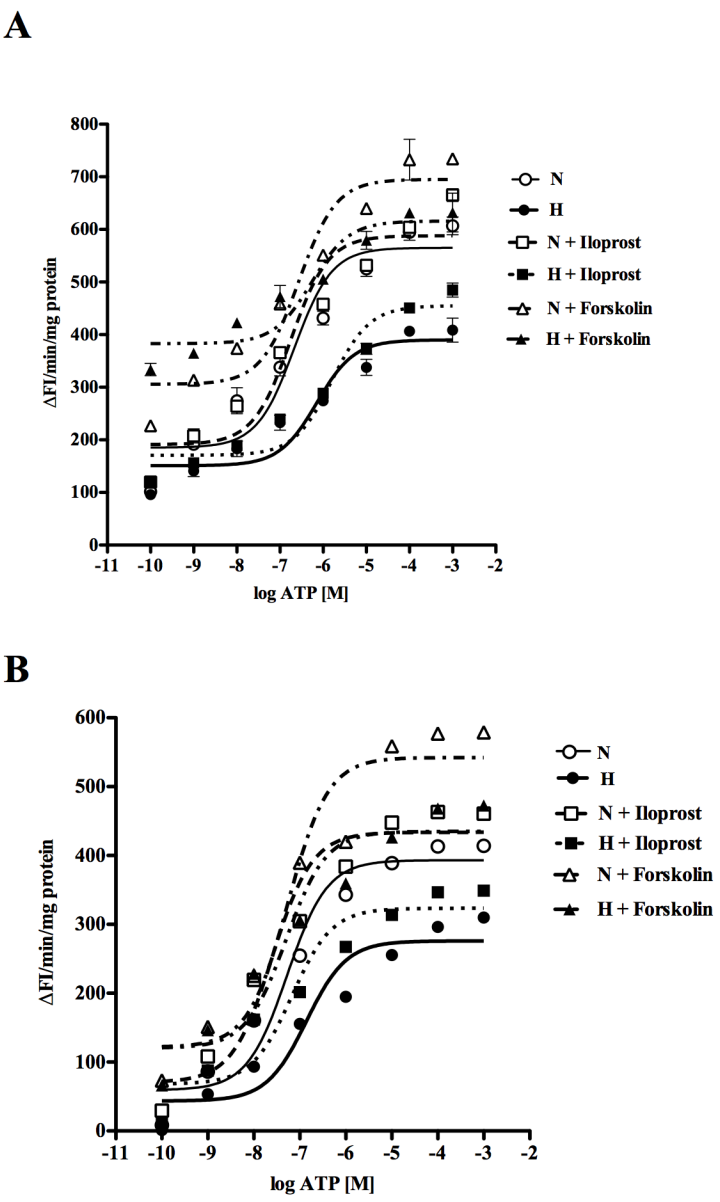


Figure 4.18 Resting and stimulated (receptor-dependent and –independent) adenylyl cyclase activity is decreased in hypoxic PASMC.

Receptor-dependent and independent AC activity measured in 72 hour hypoxic (H; 10%O_2, 5%CO_2) and normoxic (N; 21%O_2, 5%CO_2) porcine (**A**) and human (**B**) pulmonary artery myocytes, lysed following Gαs-coupled receptor stimulation with prostacyclin mimetic iloprost, or direct AC activator forskolin for 30 min prior to cell lysis. LogEC_{50} of ATP-mediated AC activation for N= -6.667, H= -6.140; N+iloprost= -6.752, H+iloprost= -5.796; N+forskolin= -6.659, H+forskolin= -6.266. For all curves, maximal AC activity higher in N myocytes compared to H ($p<0.05$).

4.5.4 Pulmonary arterial IP receptor kinetics and G-protein-coupling are paradoxically altered by hypoxia

IP receptor affinity for radioligand ^{3}H-iloprost is diminished in agonist-naïve and hypoxic pPASMC (increased dissociation constant, Kd, (**Figure 4.19A**) while IP abundance (Bmax) is unchanged in membranes (**Figure 4.19B**). Challenge with IP receptor agonist iloprost further decreases receptor ligand affinity in hypoxic myocytes, but does not affect the Kd of normoxic IP (**Figure 4.19A**). Challenge of normoxic and hypoxic pPASMCs with iloprost or AC activator forskolin followed by immediate immunoprecipitation of IP receptor and immunoblot for receptor-bound Gαs and Gαi indicate that IP coupling with Gαs is increased under hypoxia compared to normoxia **(Figure 4.19C)**. Iloprost stimulation and direct AC activation by forskolin increase IP-Gαs coupling to a similar degree in normoxia and hypoxia **(Figure 4.19C)**. IP receptor has a baseline level of association with Gαi, unchanged by stimulation or hypoxia **(Figure 4.19D).** Hypoxia does not change basal expression of Gαs and Gαi proteins.

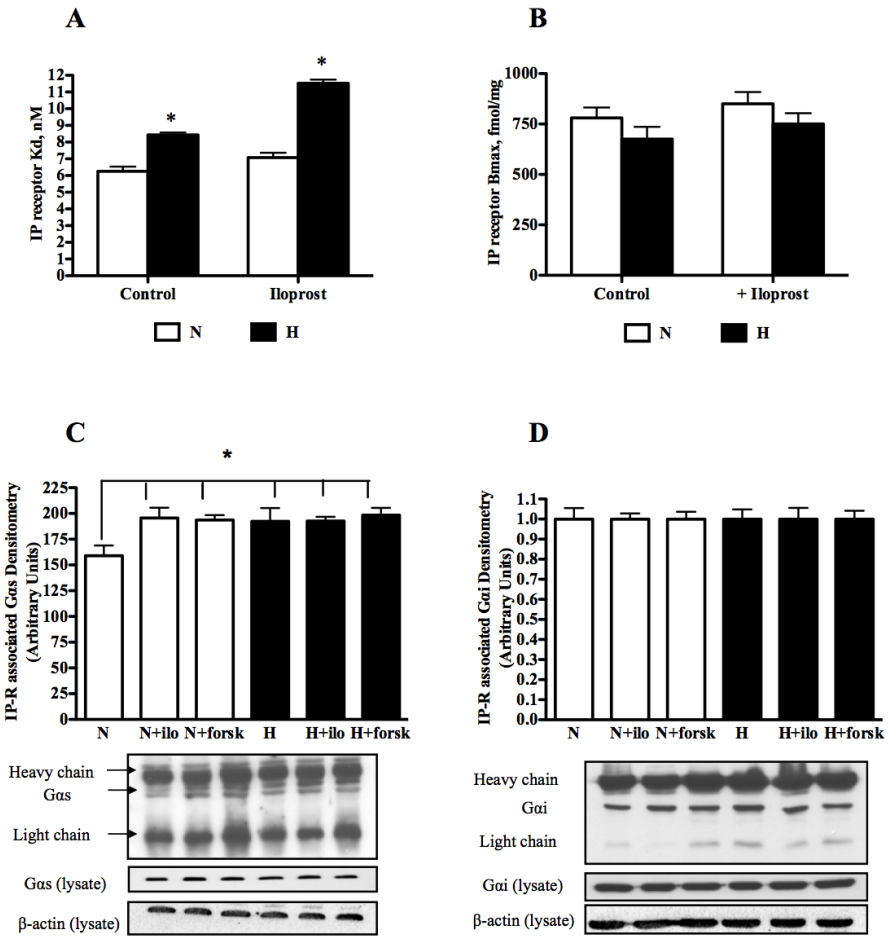


Figure 4.19 PASMC prostacyclin receptor kinetics and coupling are paradoxically altered by hypoxia.

(A) Dissociation constant (K_d) of prostacyclin receptor (IP) in hypoxic (H) and normoxic (N) agonist-naïve myocytes, and following challenge with IP agonist 1μM iloprost, by radioligand assay using ^{3}H-iloprost; higher K_d indicates decreased ligand affinity (*$p<0.05$). **(B)** Bmax (maximal binding sites, indicating membrane receptor abundance) of IP receptor in N and H, from saturation binding isotherms. **(C)** IP association with Gαs in normoxic and hypoxic cells by immunoblot following immunoprecipitation of IP receptor, after treatment with 1μM iloprost or 1μM forskolin (*; $p<0.05$ versus N, n=4). **(D)** IP association with Gαi, by immunoblot of immunoprecipitates. Whole lysate abundance of Gα proteins is unaltered.

4.5.5 AC isoform profile and abundance is unaltered by hypoxia

AC isoform mRNA expression profile in hPASMC, by RtPCR, is not changed by 72hr hypoxic exposure. Myocytes express measurable levels of message for AC 3, 6, 7, and 9 (histogram **Figure 4.20A**; representative agarose gel electrophoresis image **Figure 4.20B**. Western blots probed with pan-AC antibody reveal no change in total AC abundance following hypoxia (**Figure 4.20 C**).

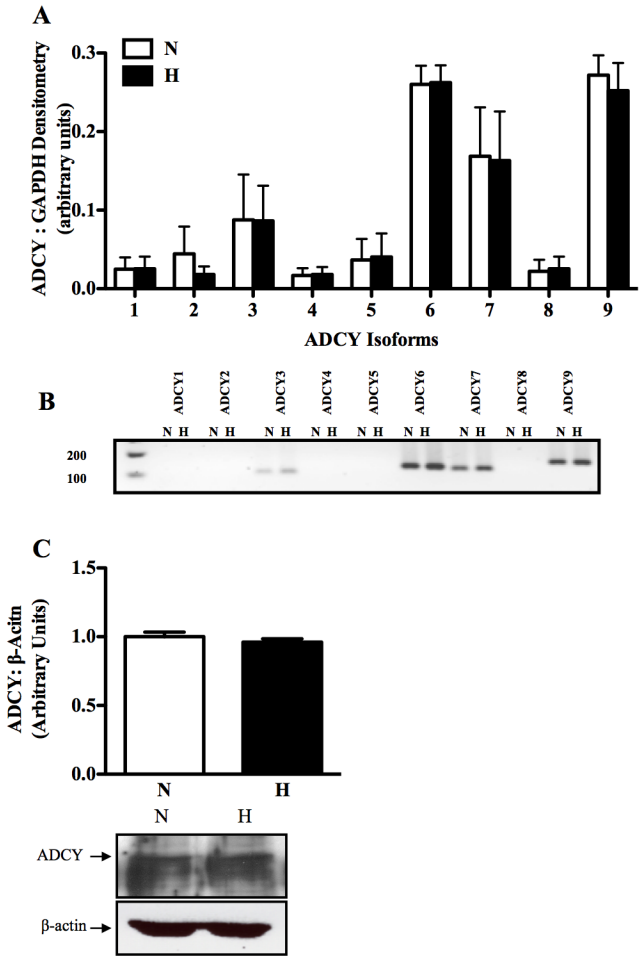


Figure 4.20 Adenylyl cyclase isoform profile and abundance in PASMCs are unaltered by hypoxia.

(A, B) mRNA expression of specific AC isoforms in hPASMC following 72hr exposure to hypoxia (H) or normoxia (N); representative agarose gel image of rtPCR products (n=3). **(C)** AC protein in whole lysates (Western blot, normalized to β-actin) in hypoxic (H) and normoxic (N) myocytes (data shown from pPASMC; n=3, p=NS).

4.5.6 cAMP and SNP-mediated relaxation is impaired in hypoxic piglet pulmonary arteries

Pulmonary artery rings from 3-day normoxic and hypoxic piglets were precontracted with 30nM thromboxane mimetic U46619 followed by dose-dependent relaxation with AC activator (forskolin) and nitric oxide donor sodium nitro prusside (SNP) were studied. Pulmonary arteries from normoxic piglets relaxed completely with either forskolin or SNP however, relaxation to forskolin and SNP was impaired in hypoxic pulmonary arteries. Forskolin log IC50: N = -5.876; H = -5.534; SNP; log IC50: N = -6.697; H = -5.125. **(Figure 4.21 A, B)**

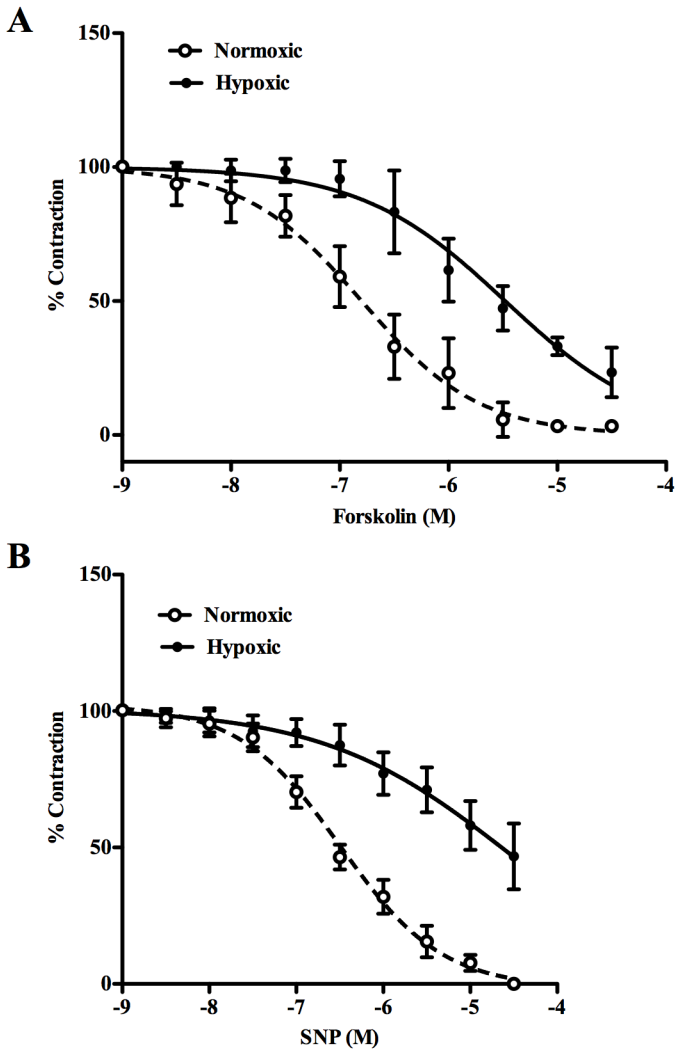


Figure 4.21 cAMP and SNP-mediated relaxation is impaired in PPHN pulmonary arteries

Pulmonary arteries from PPHN and control animals pre-contracted with 30nM U46619 (thromboxane mimetic), then relaxed with graded doses of **(A)** AC activator forskolin, or of **(B)** NO donor nitroprusside (SNP). Force of contraction normalized to KCl responses.

4.5.7 AC activity remains lower in hypoxic myocytes after Gαi inhibition by pertussis toxin or Ca^{2+} chelation by BAPTA

72 hours of hypoxic exposure decrease adenylyl cyclase activity in hypoxic myocytes compared to normoxic myocytes **(Figure 4.22 A, B)**. Treatment of pPASMCs with pertussis toxin to inhibit basal Gαi activation **(Figure 4.22 A)** and chelation of Ca^{2+} **(Figure 4.22 B)** in the lysates from hypoxic and normoxic pPASMCs by BAPTA treatment does not change AC activity significantly in the hypoxic cells compared to normoxic cells. Pertussis toxin and BAPTA treatments does not significantly decrease AC activity in the normoxic cells.

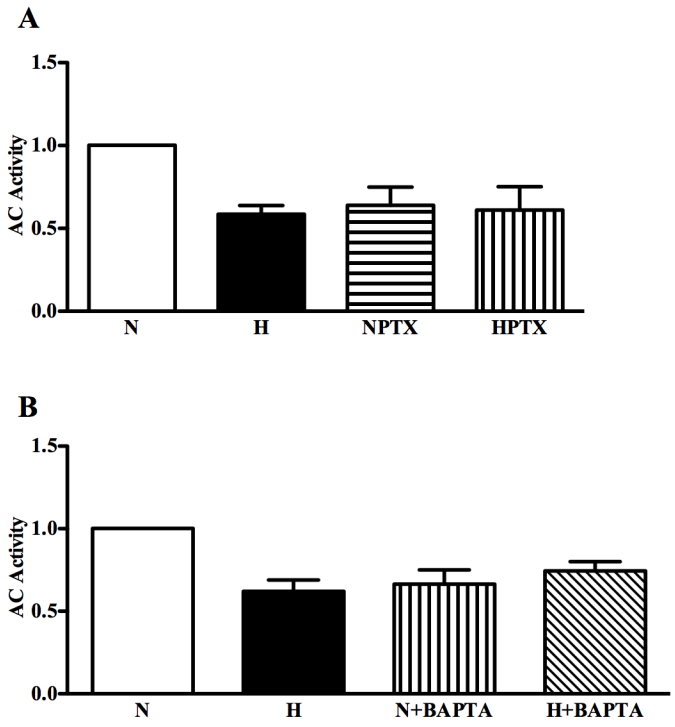


Figure 4.22 Neither pertussis toxin nor BAPTA treatment changes AC activity significantly in normoxic as well as hypoxic cells

AC activity was measured in the lysates of **(A)** normoxic and hypoxic pPASMCs treated with pertussis toxin (100ng/ml for 16 hours) or vehicle and **(B)** in the cell lysates in the presence of the Ca^{2+}-chelator BAPTA (2μM). AC activity is decreased in hypoxic pPASMCs compared to normoxic pPASMCs ($P < 0.05$). AC activity is presented as normalized to normoxic levels.

4.6 Discussion (Adenylyl cyclase study)

We conclude that [i] Pulmonary arterial hypoxia decreases smooth muscle cAMP generation and impairs the AC activator forskolin-mediated relaxant responses of U46619 precontracted pulmonary arteries from hypoxic piglets. Hypoxia decreases both agonist-naive and agonist-stimulated AC enzyme activity, observed as a decrease in velocity of product formation without change in affinity for substrate. Receptor-dependent and receptor-independent AC activity are decreased at all substrate ATP concentrations, in hypoxic myocytes. [ii] The decrease in cAMP is not due to increased degradation of cAMP, as hypoxia decreases cAMP-dependent PDE activity in proportion to diminished cAMP substrate. [618] Prostacyclin receptor affinity for agonist is decreased in hypoxia, but receptor abundance and cell surface localization are unchanged. Prostacyclin receptor coupling with Gαs is paradoxically increased under hypoxic conditions, indicating that the observed decrease in receptor-dependent AC activity may not be due to decreased IP receptor activation. [iv] AC expression is unaltered by hypoxia. The pulmonary arterial AC isoform profile prominently features AC6 and 9. [v] Basal Gαi activation is not responsible for the reduced AC activity in hypoxic PASMCs.

Hypoxia and pulmonary hypertension are associated with decreased AC pathway activity, but the locus of this downregulation has been debated. Alveolar hypoxia selectively and markedly reduces Gαs levels, reducing β_2-adrenergic signalling to cAMP [619]. Hypoxia inhibits the ability of forskolin to activate K_{Ca} channels in pulmonary artery, implying a cAMP generation defect [620]; and decreased cAMP is reported in pulmonary arterial hypoxia [42, 621]. In monocrotaline-induced pulmonary hypertension, basal and stimulated catalytic activity of AC is reduced in cardiac right ventricle and septum by 4 weeks after treatment [622]. While it has been

established that prolonged hypoxia attenuates sensitivity of pulmonary arterial AC to prostacyclin or β-adrenergic stimulation [586, 623], since no change was observed in basal AC activity, all blunted AC responses were ascribed to receptor attrition. These studies were carried out on adult rat main pulmonary artery homogenates. As prostacyclin synthesis and response [624], AC isoform expression and activity [625-627] are developmentally regulated in the neonate, it is conceivable that hypoxia has differing effects on AC activity in the neonate than the adult pulmonary artery. We previously reported that neonatal hypoxia increases Gαq coupling of the pulmonary arterial TP receptor [565], desensitizes the ligand binding ability of IP, and ablates the cAMP response to IP stimulation [596]. In the present study, we locate the defect in hypoxic neonatal AC activity at the level of enzyme regulation. Importantly, we studied AC activity in porcine PASMC, reflecting our established neonatal hypoxic PPHN model [162, 628]; and verified these data in human PASMC. ATP availability does not limit AC velocity in hypoxic myocytes, as in our preparation ATP concentration is actually increased. Resting cAMP is lower in hypoxic myocytes, and cAMP-specific PDE activity is decreased proportionally, indicating that rapid cAMP turnover does not lower basal cAMP content in hypoxia. These data confirmed the need to focus on AC activity under hypoxic conditions, rather than substrate availability or product degradation.

AC activation sequentially requires GPCR binding to agonist (in pulmonary artery, predominantly prostacyclin), interaction of the GPCR with a stimulatory Gα subunit, GTP hydrolysis, then recruitment of Gβγ subunits and cessation of signal transduction [200]. Prostacyclin is produced by myocytes in an autocrine fashion, and therefore constitutes a significant proportion of ‘resting’ AC activation [586]. We and others report that hypoxia does

not diminish pulmonary arterial myocyte production of prostacyclin, though it increases thromboxane to a greater degree [179, 629, 630]. IP receptor expression is unchanged by hypoxia, even while the IP-mediated cAMP response is lost [596]. Here we find, while IP affinity for agonist is decreased in hypoxia, a compensatory increase in IP association with Gαs protein. Cell membrane IP is unaltered after 72h hypoxia, indicating receptor turnover or tachyphylaxis may not be important in this time frame. We therefore conclude that while hypoxia may diminish IP ligand affinity, it does not cause receptor uncoupling; and that a loss of IP signal is not the sole cause of AC down-regulation and loss of cAMP in hypoxic pulmonary arterial myocytes.

The AC assay method using loss of lanthanide luminescence of terbium norfloxacin is specific, linear with ATP to cAMP conversion, and not damped by presence of cAMP or adenosine monophosphate (AMP) [595]. We measured AC velocity against time and substrate gradients, in membrane fractions clear of interfering proteins, and calculated the kinetics of hypoxic versus normoxic AC enzyme activities in presence and absence of stimulatory agonists. Hypoxic AC has a Km similar to that of normoxic AC, but lower velocity of product formation per time, suggesting that the AC enzyme is not structurally altered by hypoxia, but that its regulation is altered, possibly at the level of its interaction with GPCR-G-protein complexes.

We then examined whole cell AC catalytic function, in human and porcine PASMC lysates obtained following live myocyte stimulation with agonist for IP receptor, a vasodilator GPCRs abundantly expressed in pulmonary artery and coupled to Gαs. A limitation of this methodology is the ability to maintain AC activation state during sample preparation; agonist-stimulated cells

may not exhibit the height of AC activation, compared to agonist-naïve cells, as could be measured in live cell assays. However we are able to demonstrate a limited degree of agonist-responsiveness of substrate-dependent AC activation in normoxic myocytes, and a lower response in hypoxic myocytes at all substrate concentrations. Direct AC activation by forskolin occurs in hypoxic myocytes, but never to equal the AC activity elicited in normoxic cells under similar stimulation. We propose that the loss of hypoxic AC activity is a phenomenon downstream of receptor function, centered on regulation of AC.

AC activation is tightly regulated, as intracellular cAMP activates many downstream pathways. Localization of AC isoforms to distinct lipid raft domains or sub-cellular regions determines the regulation of distinct cAMP pools [631, 632]. We report that hypoxia does not change the expression profile of pulmonary arterial AC isoforms. AC isoforms are differentially up- or down-regulated by signals from G proteins (activated by Gαs; inhibited by Gαi and Gβγ subunits), PKA and PKC, calmodulin and intracellular Ca^{2+} [633]. AC 5 and 6 are predominant cardiac isoforms [634, 635]; AC isoforms 1, 4, 7 and 8 localize to the bronchial epithelium, and AC 2, 3 and 5/6 are expressed in pulmonary artery [634, 636]. While intracellular Ca^{2+} concentrations below 50nM are not known to regulate most ACs [637], a physiological increase in Ca^{2+} inhibits AC 5 and 6, resulting in a negative feedback loop shutting down Gαs signaling [200]. Ca^{2+}-sensitive AC isoforms are abundant in heart and pulmonary artery, and AC6 is increased in fetal and neonatal tissues [633, 634]. Newborns may thus be more sensitive to inhibitory effects of Ca^{2+} on AC activity. While unable to distinguish protein abundance of AC isoforms, at the level of mRNA we observe myocytes prominently expressing 6 and 9, with AC3 and 7 as minor components. Lacking available porcine AC primers, this was studied in human

PASMCs. Both AC7 and 9 are activated by Gαs; AC9 is relatively insensitive to regulation (including by forskolin). AC6 is sensitive to inhibition by Gαi and intracellular Ca^{2+} in the μM range [200].

We have previously shown that newborn swine PASMCs have increased resting Ca^{2+} levels [159]. To investigate the role of basal Gαi signaling and elevated calcium in the regulation of AC activity, we performed an AC activity assay in the presence of Gαi inhibition (PTX treatment) and Ca^{2+}-chelation by BAPTA treatment. However, neither PTX nor BAPTA treatment normalized AC activity in hypoxic PASMCs to normoxic levels suggesting that basal Gαi signaling and elevated Ca^{2+} levels may not be etiologic in the decreased AC activity seen in hypoxic PASMCs. In rat left ventricular cardiomyocytes, PTX treatment inhibits basal Gαi activity and shifts the balance towards Gαs, leading to more AC activation and inotropic responses [638]. However, similar to our observations in newborn swine PASMCs, PTX treatment in adult rat PASMCs did not increase basal cAMP levels although cicaprost-stimulated cAMP levels were decreased in the presence of PTX treatment [639]. This suggests that basal Gαi activity might not play a major role in AC inhibition in rats and newborn swine pulmonary arteries. At present, we have studied the effect of PTX treatment in unstimulated conditions only; as observed in rat PASMCs, Gαi could be involved in AC inhibition only under Gαi stimulated conditions in newborn swine PASMCs. To obviate the non-specific or lethal effects of BAPTA on cultured PASMCs during hypoxic and normoxic exposure, we studied AC activity in BAPTA-treated lysates. Calcium-inhibitable AC isoforms AC5 and AC6 are mainly susceptible to calcium entering into the cell from capacitative Ca^{2+} entry [640] and since resting calcium levels in newborn swine PASMCs are nifedipine-sensitive [159] it is possible that the inhibition

of L-type calcium channels might be more effective to study the possible role of Ca^{2+} in AC6 inhibition in PASMCs under hypoxic conditions.

The impairment of NO donor-induced vasorelaxation of U46619 precontracted pulmonary arteries from 3-day hypoxic piglets observed in this study is consistent with other studies such as norepinephrine precontracted pulmonary arteries from duct ligated PPHN lambs [641, 642] and U46619 and ET-1 precontracted pulmonary arteries from chronic hypoxic piglets exposed to 3 or 10 days of *in vivo* hypoxia [643], all of which show impaired relaxations to an NO donor. However, PGF2α precontracted pulmonary arteries from hypoxic piglets are reported to show normal relaxation to NO donor [323]. We also observed impaired relaxation to the AC activator forskolin in hypoxic pulmonary arteries compared to normal pulmonary arteries. However, forskolin-induced relaxation is not reduced in norepinephrine precontracted pulmonary artery rings from duct ligated fetal lambs although relaxation to iloprost and prostacyclin is reduced [644], suggesting that in the duct ligation model, an AC defect might occur at the receptor-G-protein level. Furthermore, in an endothelin precontracted cannulated artery preparation study from piglets (2-3 day old) exposed to hypoxia (8-10% up to 3-4 days) prostacyclin-stimulated relaxation were not significantly altered compared to normoxic piglets [155]. We conclude that the degree of measured relaxation impairment in PPHN arteries may be influenced by the nature of the arterial preparation; in particular the pre-contracting agent; but that there is precedent for our observation of impaired forskolin-mediated relaxation of force generation, in hypoxic PPHN arteries.

In this study, we found that IP receptor affinity is moderately decreased, but no change in the cell surface abundance of IP receptors occurs due to hypoxia. Moreover our co-immunoprecipitation

results suggest that the IP receptor is more associated with Gαs in hypoxic conditions, suggesting a relative increase in receptor-G-protein coupling. We conclude that the reduced relaxation response of forskolin in the hypoxic pulmonary artery could be due to decreased AC activity.

This study sheds some light on a major signalling pathway in hypoxic PPHN, where loss of function can worsen pulmonary vasoconstriction in the neonate. Global defects in the AC pathway could explain the tandem clinical observations of pulmonary vasoconstriction and loss of cardiac contractility in PPHN; examination of AC catalytic function in hypoxic neonatal cardiomyocytes is also warranted. We are currently pursuing specific mechanisms for the observed AC downregulation. Hypoxic attenuation of pulmonary arterial AC activity could be amenable to treatment by targeted direct adenylyl cyclase enzyme activators, to facilitate signalling by receptors linked to AC and thus restore vasodilator and possibly inotropic responses in PPHN.

5 Overall Conclusions

Persistent pulmonary hypertension of the newborn (PPHN) results from the failure of pulmonary vascular resistance to decrease at birth, due to perinatal hypoxia, inflammation or direct lung injury. PPHN is characterized by an elevated thromboxane to prostacyclin ratio, and pulmonary hyperreactivity and hypersensitivity. Thromboxane also plays a pivotal role in septic pulmonary hypertension in newborns. Pulmonary vasoconstrictive effects of thromboxane are mediated through thromboxane receptor (TPα) coupling with a G-protein Gαq.

Regulation of thromboxane receptor signaling activity occurs at the level of the receptor-G-protein complex. We hypothesized that a series of post-translational modifications of TP receptor and Gαq protein serve to regulate critical interactions between these moieties, and also regulate receptor-ligand binding and signaling ability; and that these important post-translational modifications are sensitive to hypoxia of the neonatal pulmonary circuit. Post-translational modifications are known to play a seminal role in protein-protein interaction as well as trafficking of proteins to the cell membranes. The major post-translational modification regulating TPα signaling is phosphorylation, whereas palmitoylation plays a significant role in trafficking and interaction of Gαq with upstream GPCRs.

5.1.1 Gαq palmitoylation

We studied the role of Gαq palmitoylation in the TPα-mediated hyper-reactive response of PA rings from hypoxic PPHN and control piglets, also seen in pulmonary artery and aortic myocytes from newborn swine. We studied the role of site-specific palmitoylation of candidate cysteine residues on Gαq, in TP-stimulated Ca^{2+}-mobilization. Our results suggest that under

hypoxic conditions, palmitoylation of Gαq increases, without altering cellular palmitate uptake relative to protein synthesis. Pharmacological inhibition of palmitoylation dose-dependently impairs TP-stimulated Ca^{2+} mobilization in pulmonary arterial myocytes, but does not affect TP-stimulated Ca^{2+} mobilization in hypoxic aortic myocytes, indicating the increased Gαq palmitoylation may facilitate TP hyper-reactivity specifically in the pulmonary circuit. Site-directed mutagenesis studies of palmitoylable cysteine residues of Gαq in HEK293 cells reveal that whereas one cysteine is sufficient for TP-Gαq interaction, both cysteines are required for efficient TP-Gαq-mediated Ca^{2+} mobilization. Mutation of both the palmitoylable cysteines of Gαq abolishes membrane presence of Gαq, and is associated with a severe decrease in TP-stimulated Ca^{2+} mobilization. Pharmacological inhibition of palmitoylation dose-dependently decreases TP-stimulated calcium mobilization in HEK293T cells. Our overall conclusion is that Gαq palmitoylation increases in hypoxic pulmonary artery myocytes, contributing toward thromboxane-mediated hyper-reactivity in PPHN arteries. The apparent specificity of this finding to the pulmonary circuit is promising; however, the non-specificity of available palmitoylation inhibitors prohibits these agents from use as potential pharmacological tools to treat pulmonary arterial hyperresponsiveness in PPHN.

5.1.2 TP phosphorylation

TPα is the major isoform present in vascular smooth muscle and is regulated by phosphorylation of C-terminal serine residues by various kinases, leading to desensitization. We found that in hypoxic PPHN animals, TP serine phosphorylation is reduced, and this is associated with TP hyper-reactivity and hypersensitivity observed in pulmonary artery rings from hypoxic piglets. Studies on HEK293 cells suggested that hypoxia decreases PKA but not PKC activity. Thus

hypoxia, by decreasing PKA activity, decreases TP desensitization. Within these studies, we were able to restore TP phosphorylation by incubation with milrinone, a PDE3 inhibitor, implying that the state of TP phosphorylation may be treatable pharmacologically. Finally from site-specific mutation of TP serines, we determined the locus of regulation by this key post-translational modification of the TP protein. Site-directed mutagenesis of C-terminal serine residues revealed that serine 324 is the primary target of PKA-mediated phosphorylation and is involved in regulation of TP receptor-agonist interactions. Ser329A mutant of TPα is no different from wild-type TPα receptors. Ser331A is insensitive to hypoxia and PKA with a decreased agonist-mediated response. Ser331 mutation confers loss of TP receptor-agonist interaction, regardless of PKA activity. Overall these studies confirm the impact of site-specific PKA-mediated phosphorylation on TP receptor-ligand interaction, and the importance of this axis in regulating pulmonary arterial TP activity.

5.1.3 Adenylyl cyclase activity in hypoxic PPHN

Adenylyl cyclase enzymes are responsible for the generation of cAMP. Normal levels of cAMP are essential for the maintenance of physiological pulmonary vascular tone, PASMC proliferation as well as cardiac contractility**.** After discovering that hypoxia diminishes pulmonary arterial PKA activity, resulting in unchecked TP-mediated vasospasm, we investigated the mechanism by which upstream signaling of Gαs-coupled vasodilators through cAMP to activate PKA may be impaired by hypoxia. We found that PASMCs exposed to hypoxia have decreased adenylyl cyclase activity compared to normoxic controls, resulting in limited cAMP generation upon Gαs-coupled receptor stimulation. Our results suggest that hypoxia-induced decrease in basal as well as receptor stimulated AC activity in PASMCs is

neither due to poor substrate (ATP) availability, nor increased degradation of cAMP. IP receptor coupling with G-protein Gαs is not decreased. We also found that inhibition of basal Gαi activation is not capable of normalizing AC activity in hypoxic PASMCs to the normoxic levels. AC enzyme kinetics suggest that AC structure is not altered by hypoxia (as Kd is unchanged), but that the velocity of ATP catalysis to cAMP is slowed. We conclude that signaling of the receptor-Gαs complex to activate AC is not impaired by hypoxia, and inhibitory Gαi signaling is not elicited by hypoxia; but speculate that hypoxia may directly impair AC activity. A mechanistic investigation of the effect of hypoxia on AC catalytic velocity is ongoing.

Our studies suggest that AC6, AC7 and AC9 are the major isoforms of adenylyl cyclase enzyme present in human PASMCs. Of these, AC7 and AC9 are Ca^{2+} insensitive isoforms, whereas Ca^{2+} inhibits the enzyme AC6. There is evidence in the literature that Ca^{2+} entering through capacitative Ca^{2+} entry inhibits AC6. Hypoxic PASMCs have elevated Ca^{2+} levels, suggesting susceptible AC activity may be diminished. Chelation of Ca^{2+} in the PASMCs lysates has no effect on total AC activity; however we speculate that calcium entry through L-type Ca^{2+} channels might selectively inhibit AC6 under hypoxic conditions, and propose examination of the effect of calcium channel blockade on the activity of hypoxic AC6.

5.1.4 Integrated conclusions

Taken together, we find that site-specific palmitoylation and phosphorylation are critical for well-regulated function of proteins in the receptor-G-protein complex, and that these post-translational modifications are susceptible to hypoxia – palmitoylation of Gαq increases, and phosphorylation of TP decreases – contributing to increased pulmonary arterial vasoconstriction under hypoxic conditions.

Potential pharmacological solutions to these problems are currently limited, and require some speculation. Gαq palmitoylation is required for trafficking to the cell membrane and interaction with TPα; however in hypoxic pulmonary artery, increased palmitoylation of Gαq contributes to TPα hyperreactivity. Regulation of palmitoyl acyl transferase enzymes (DHHC proteins) is not studied in pulmonary circuit, nor are specific inhibitors of DHHC proteins available to test their possibility as a drug target in hypoxic pulmonary hypertension. Non-targeted inhibition of palmitoylation may confer significant side-effects, as so many key cellular proteins undergo palmitoylation. Therefore this remains a topic for future investigation. TPα phosphorylation through Gαs-coupled receptors plays a vital role in desensitization of TPα and keeps TPα-mediated vasoconstriction at physiological levels. Though phosphodiesterase inhibitors are available to inhibit degradation of cAMP, reduced AC activity under hypoxic conditions may limit their therapeutic effects, due to reduced cAMP formation. Strategies to increase cAMP formation (for example, selective adenylyl cyclase activators) may thus be required to decrease abnormally elevated PVR as well as to increase cardiac contractility and tissue oxygenation in PPHN patients.

6 Future Directions

Future directions for the research in this thesis are sorted according to the major post-translational mechanisms we have identified which enact regulation of the receptor-G-protein complex, and discuss some remaining gaps in our current understanding of these processes. We will focus on identifying potential pharmaceutical targets that could promote pulmonary vasodilation in persistent pulmonary hypertension of the newborn.

6.1.1 G-protein palmitoylation

Protein palmitoylation is a unique lipid modification of proteins, due to its reversibility and dynamicity[645]. Initial research on GPCR and G-protein palmitoylation in various cell lines revealed that most of the GPCRs and G-proteins are palmitoylated, and that palmitoylation plays a major role in signaling and trafficking of these proteins. Despite this, the regulation[605, 646] of G-protein and GPCR palmitoylation is largely unstudied in primary cells or tissues from normal or hypertensive animals.

It is long known that the majority of G-protein alpha subunits are palmitoylated at their N-terminus; however, for most of the G-proteins candidate palmitoylation enzymes are not known. Candidate palmitoylation enzymes for G-protein Gαq, Gαs and Gαi2, are studied in HEK293 cells[566] but their regulation under normal and hypoxic conditions in vascular smooth muscle in not studied. Most of the Gαq coupled GPCRs also couple with Gα12/13 G-proteins to initiate sensitization of contractile apparatus[388]. Identification of Gα12/13 palmitoylation enzymes and their regulation in hypoxic conditions might reveal new insights to understand calcium sensitization and actin polymerization.

6.1.2 GPCR palmitoylation

Maintenance of normal pulmonary vascular tone is the result of a balance between vasoconstrictor and vasodilator GPCRs signaling. Though most of the GPCRs (e.g. endothelin, 5-HT, TPβ adenosine, prostacyclin and bradykinin B2) are known to be palmitoylated, their candidate palmitoylation enzymes (DHHC proteins) are not known at present. Studying the regulation of DHHC proteins in normal and hypertensive animals will shed light upon the pathophysiology of hypertensive disorders and might help us to target these DHHC proteins therapeutically.

6.1.3 Redox regulation of GPCR and G-protein palmitoylation

Reactive oxygen species are well known for their deleterious effects in asthma and pulmonary hypertension [647] and [621]. Interestingly, reactive oxygen species such as H_2O_2 decrease palmitoylation of other proteins such as caveolin-1 and H-Ras in bovine endothelial cells and CD81 in Jurket cells [648], [649] and [650]. In addition, nitric oxide (NO) also inhibits both basal and isoproterenol-induced increases in (^{3}H) palmitate uptake in β_2–adrenergic receptors leading to functional uncoupling of the receptor and G-protein α [651]. Consistent with this observation, NO could inhibit palmitoylation of other GPCRs and proteins including endothelial and inducible nitric oxide synthase to affect caveolar localization of endothelial nitric oxide synthase [652] and trafficking and activity of inducible nitric oxide synthase [653], suggesting NO might, at least in part, regulate its own production. S-nitrosylation of cysteine residues is another mechanism by which NO can directly interfere with palmitoylation by making cysteines unavailable for palmitate [654]. Clearly, in-depth investigation of redox regulation of GPCR and G-protein palmitoylation seems warranted.

6.1.4 Adenylyl cyclase

We concluded that regulatory TP phosphorylation is decreased in hypoxic pulmonary artery myocytes, as a direct result of decreased PKA activity owing to decreased cAMP generation. This leads to TP receptor hypersensitivity and hyperreactivity, and thus unmitigated pulmonary arterial contraction. General TP antagonists have been studied in pulmonary hypertension, but due to side-effects of decreased platelet adherence, are impractical for clinical use. Thus, the most reliable method of attenuating TP-mediated vasoconstriction and promoting pulmonary vasodilation would be to restore cAMP-mediated regulation of pulmonary arterial TP receptors. We have already studied one approach to this, the inhibition of cAMP degradation by use of milrinone. Milrinone is currently used as a second or third line agent in the clinical treatment of neonatal pulmonary hypertension, in part for its inotropic effect on cardiac contraction, and in part for its action as a pulmonary vasodilator (a secondary mechanism of action delineated mainly by the work of our lab). So any further examination of this agent would occur henceforth in the context of clinical trials.

The other practical approach to restoring the balance of TP/IP signaling in the hypoxic pulmonary circuit would be to restore the activity of adenylyl cyclase. Hypoxia decreases AC activity in hypoxic pulmonary artery smooth muscle cells; however, at present, we do not have a mechanism by which hypoxia induces decreased AC activity. Since PASMCs have higher levels of cytosolic Ca^{2+} under hypoxic conditions, it is possible the increased basal Ca^{2+} may inhibit the activity of AC6 (the AC isoform most abundantly expressed in PASMCs), as this isoform is known to be inhibited by calcium entering into the cells by capacitative Ca^{2+} entry. However, the role of capacitative Ca^{2+} entry in inhibition of AC6 in newborn PASMCs is not yet studied. There is evidence in the literature that AC6 enzyme partitions primarily into lipid rafts and

caveolae of cardiac myocytes [655], and aortic smooth muscle cells [656]. Absence of AC from lipid rafts could be a potential mechanism by which hypoxia may diminish the biological activity of AC. However, distribution of AC6 into lipid rafts and caveolae has not been studied in newborn pulmonary artery smooth muscle cells under normoxic and hypoxic conditions. Effects of hypoxia on expression levels and localization of AC6 in PASMCs should be studied by using AC6 specific antibodies. Finally, we propose to study the redox regulation of AC6. There is evidence that protein nitration may regulate activity of this key enzyme. Nitric oxide inhibits AC6 activity in neuroblastoma cells [657] as well as in cardiac myocytes and pulmonary endothelial cells [631] whereas the possible effect of nitric oxide on AC6 activity is not known in newborn PASMCs. Importantly, as nitric oxide is a first-line agent for the treatment of pulmonary hypertension, any inhibitory effects of nitric oxide on adenylyl cyclase activity (especially in the context of hypoxia) may prove critical. Once a specific mechanism can be identified for down-regulation of AC activity in hypoxic PASMC, it may become possible to target this mechanism.

7 Supplemental Figures

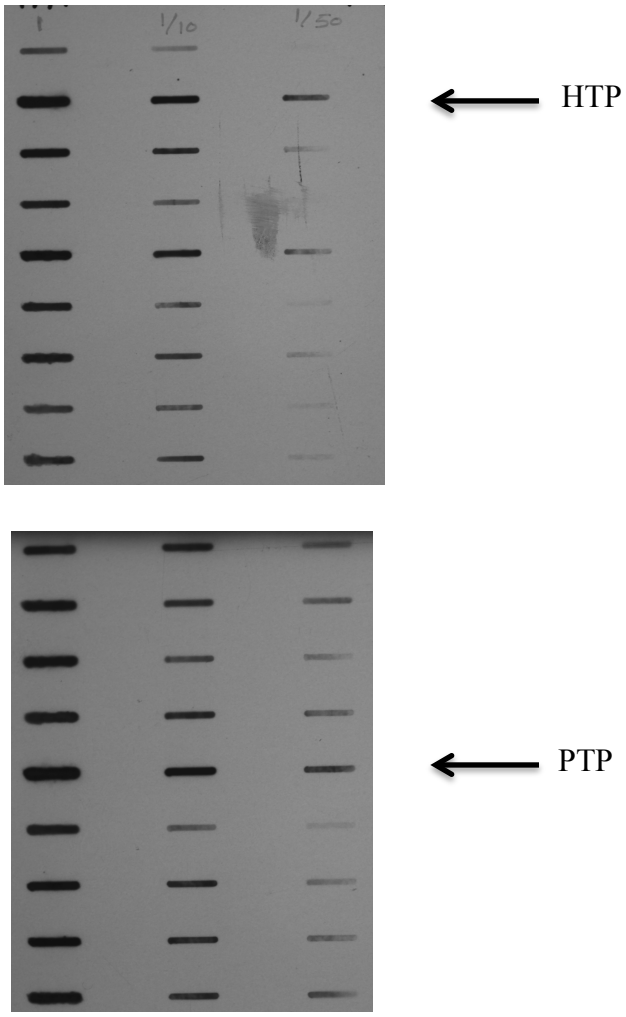
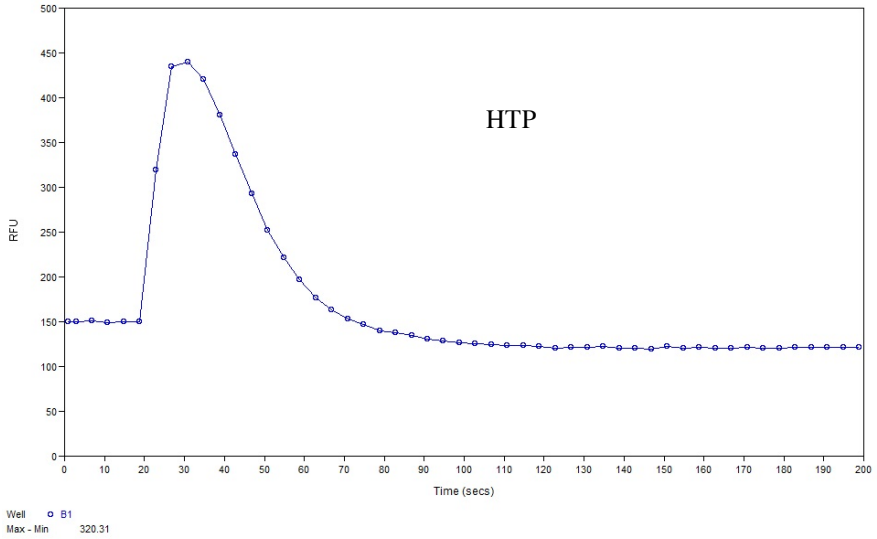


Figure 7.1 Slot blot of HTP and PTP clones.

Cells were lysed in lysis buffer and lysates were loaded on to slot blot apparatus having nitro cellulose membrane in serial dilutions of 1, 1/10, and 1/50 followed by suction of lysates by a vacuum. Membranes were probed for Rho 1D4 antibody. Clones were selected on the basis of proportionate decrease in signal and showing maximum signal at highest dilution of (1/50). Clones selected for Ca^{2+} assay and characterization are shown by arrows.

A



B

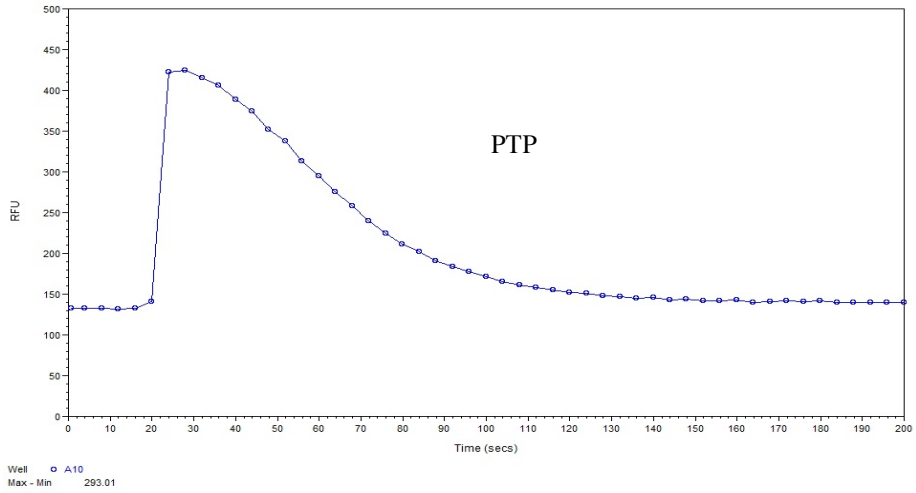


Figure 7.2 Characterization of HTP and PTP stable cell lines

HEK293T cells stably expressing human (HTP) and porcine (PTP) thromboxane receptor were loaded with calcium sensitive dye fluo-4NW followed by calcium assay as described in method section. Cells were stimulated with 10^{-6}M U46619. X axis shows the relative fluorescence unit (RFU) and Y axis shows time in seconds.

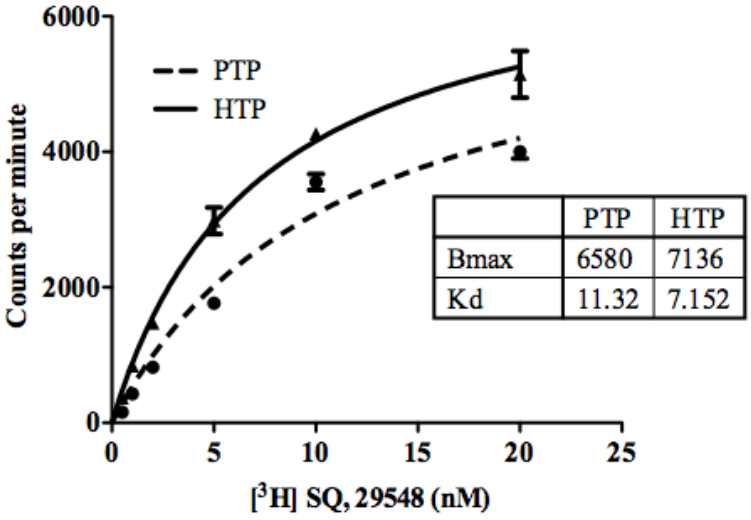
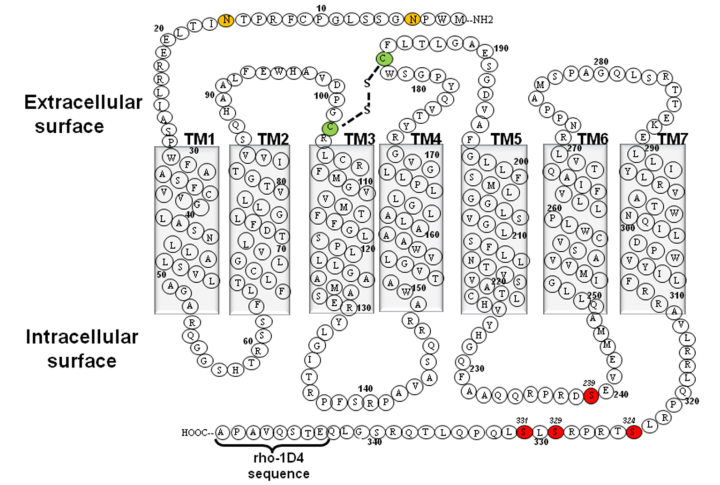


Figure 7.3 Saturation binding assay of membrane bound HTP and PTP from HEK293 stable cell lines using radioligand [^{3}H] SQ29548.

Saturation binding assay was done as described in the materials and methods.




```
Human_TP_alpha          MWPNGSSLGPCFRPTNITLEERRLIASPWFAASFCVVGLASNLLALSVLA 50
Human_TP_beta           MWPNGSSLGPCFRPTNITLEERRLIASPWFAASFCVVGLASNLLALSVLA 50
PorcineTHY01_0087_E01   MWPNGSSLGPCFRPTNITLEERRLIASPWFAASFCLVGLASNLLALGVLA 50
                        ************************************:**********.*** 

Human_TP_alpha          GARQGGSHTRSSFLTFLCGLVLTDFLGLLVTGTIVVSQHAALFEWHAVDP 100
Human_TP_beta           GARQGGSHTRSSFLTFLCGLVLTDFLGLLVTGTIVVSQHAALFEWHAVDP 100
PorcineTHY01_0087_E01   GARQSSSYGRSSFLTFLCGLVLTDFMGLLSTGAIVVSQHAALFDWQAVDP 100
                        ****..*: ****************:*** **:**********:*:**** 

Human_TP_alpha          GCRLCRFMGVVMIFFGLSPLLLGAAMASERYLGITRPFSRPAVASQRRAW 150
Human_TP_beta           GCRLCRFMGVVMIFFGLSPLLLGAAMASERYLGITRPFSRPAVASQRRAW 150
PorcineTHY01_0087_E01   SCRLCHFMGVIMVFFGLCPLLLGAAMASERFLGITRPFSRPTATSHRRAW 150
                        .****:****:*:****.***********:**********:.:*:**** 

Human_TP_alpha          ATVGLVWAAALALGLLPLLGVGRYTVQYPGSWCFLTLGAESGDVAFGLLF 200
Human_TP_beta           ATVGLVWAAALALGLLPLLGVGRYTVQYPGSWCFLTLGAESGDVAFGLLF 200
PorcineTHY01_0087_E01   AMVGLVWATALVLGLLPLLGVGRYTVQYPGSWCFLTLGSEPGDVAFGLLF 200
                        * ******:**.*****************************:*.********* 

Human_TP_alpha          SMLGGLSVGLSFLLNTVSVATLCHVYHGQEAAQQRPRDSEVEMMAQLLGI 250
Human_TP_beta           SMLGGLSVGLSFLLNTVSVATLCHVYHGQEAAQQRPRDSEVEMMAQLLGI 250
PorcineTHY01_0087_E01   ACFGSLSVGLSFLLNTISVATLCFVYHGQETAQQRPRDCEVEMMAQLMGI 250
                        : :*.***********:******.******:*******.********:** 

Human_TP_alpha          MVVASVCWLPLLVFIAQTVLRNPPAMSPAGQLSRTTEKELLIYLRVATWN 300
Human_TP_beta           MVVASVCWLPLLVFIAQTVLRNPPAMSPAGQLSRTTEKELLIYLRVATWN 300
PorcineTHY01_0087_E01   MVVASICWMPLLVFIAQTVLRSPPVMSLTGQLPRATEQQLLIYLRVATWN 300
                        *****:**:************.**.** :***.*:**::********** 
                                              S324 S329 S331
Human_TP_alpha          QILDPWVYILFRRAVLRRLQPRLSTRPR-SLSLQPQLTQR--------- 339
Human_TP_beta           QILDPWVYILFRRAVLRRLQPRLSTRPRRSLTLWPSLEYSGTISAHCNLR 350
PorcineTHY01_0087_E01   QILDPWVYILFRRAVIRRLHPRLSTRSR-SLSLQPQLSRR--------- 339
                        ***************:***:******.* **:* *.* 

Human_TP_alpha          -------------------------------------SGLQ--------- 343
Human_TP_beta           LPGSSDSRASASRAAGITGVSHCARPCMLFDPEFDLLAGVQLLPFEPPTG 400
PorcineTHY01_0087_E01   -------------------------------------PTMQ--------- 343
                                                             . :* 

Human_TP_alpha          -------
Human_TP_beta           KALSRKD 407
PorcineTHY01_0087_E01   -------
```

Figure 7.4 Protein sequence alignment of porcine TP and human TP receptor isoforms.

2D structure of human TPα, indicating position of four serines targeted for alanine substitution. Comparative sequence alignment of human TP isoforms, TPα and TPβ, with porcine TP; S324, S329 and S331 are the only phosphorylatable C-terminal residues common to human TPα and porcine TP (but not TPβ).

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