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Characterization of Protein Kinase C Alpha Deficiency in a Mouse Model

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Contents

List of Abbreviations

5-HTT	**5**-**H**ydroxy**t**ryptamin**T**ransporter
AAAS	**A**chalasia, **A**drenocortical insufficiency, **A**lacrimia **S**yndrome
Add1	α **Add**ucin **1**
ATM	sodium **a**uro**t**hio**m**alate hydrate
BIM	**Bi**sindolyl**m**aleimide I
BK_{Ca}	Large (**B**ig) Conductance **Ca**$^{2+}$ activated **K**$^{+}$ channels
Ca^{2+}-CaM	**Ca**lcium-**Ca**l**m**odulin complex
CaM	**Ca**l**m**odulin
cGMP	**c**yclic **G**uanosine **M**ono**p**hosphat
CPI-17	**C**-Kinase-activated **P**rotein phosphatase-1 **I**nhibitor, **17**kDa
DAG	**Di**a**g**lycerol
DMSO	**Di**m**s**ulf**o**xid
EB	**E**sophageal **B**ody
EC	**E**ndothelial **C**ells
ET-1	**E**ndo**t**helin-**1**
ET_A	**E**ndo**t**helin-1 type **A** receptor
ET_B	**E**ndo**t**helin-1 type **B** receptor
et al.	**et al**ii (Latin for “and others”)
GDNF	**G**lia cell line-**d**erived **N**eutrophic **F**actor
HPV	**H**ypoxic **P**ulmonary **V**asoconstriction
IC_{50}	Half maximal **I**nhibitory **C**oncentration
IPML	**I**solated **P**erfused and ventilated **M**ouse **L**ung
K_{ATP}	**ATP**-sensitive **K**$^{+}$ channels
KCNK	Two-pore-domain potassium (**K**$^{+}$) **C**hannel or Potassium (**K**$^{+}$) **C**hannel subfamily **K**
K_V	**V**oltage-gated **K**$^{+}$ channels
LCM	**L**aser **C**apture **M**icrodissection
LES	**L**ower **E**sophageal **S**phincter
MLC	**M**yosin **L**ight **C**hain
MLCK	**M**yosin **L**ight **C**hain **K**inase
MLCP	**M**yosin **L**ight **C**hain **P**hosphatase
mRNA	**m**essenger **R**ibo**n**ucleic **A**cid
NO	**N**itric **O**xide
nNOS	**n**euronal **N**itric **O**xide **S**ynthase

P	**P**ressure
PAH	**P**ulmonary **A**rterial **H**ypertension
PASMC	**P**ulmonary **A**rterial **S**mooth **M**uscle **C**ell
PDE-5	**P**hospho**die**sterase type **5**
PGI2	**P**rosta**g**landin **I2**
PH	**P**ulmonary **H**ypertension
aPKC	**a**typical **P**rotein **K**inase **C**
cPKC	**c**onventional **P**rotein **K**inase **C**
nPKC	**n**ovel **P**rotein **K**inase **C**
PKC	**P**rotein **K**inase **C**
PKCα	**P**rotein **K**inase **C alpha**
PKCβ	**P**rotein **K**inase **C beta**
PKCγ	**P**rotein **K**inase **C gamma**
PKCδ	**P**rotein **K**inase **C delta**
PKCε	**P**rotein **K**inase **C epsilon**
PKCθ	**P**rotein **K**inase **C theta**
PKCη	**P**rotein **K**inase **C eta**
PKCμ	**P**rotein **K**inase **C my**
PKCι/PKCλ	**P**rotein **K**inase **C iota/lambda**
PKCζ	**P**rotein **K**inase **C zeta**
PLC	**P**hospho**l**ipase **C**
Q	Flow
ROC	**R**eceptor-**O**perated **C**hannel
s	**S**econds
SOC	**S**tore-**O**perated **C**hannel
TASK-1	**T**WIK-related **A**cid-**S**ensitive $\mathbf{K}^{+}$ channel **1**
TGF-ß	**T**ransforming **G**rowth **F**actor beta
Th2	**T**-**h**elper cell type **2**
TXA2	**T**hrombo**x**ane **A2**
VSMC	**V**ascular **S**mooth **M**uscle **C**ell
VOC	**V**oltage-**O**perated **C**hannel
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
WT	**W**ild**t**ype

1 Introduction

Protein Kinase C alpha (PKCα) is a widely expressed signaling molecule in the mammalian organism. Its role in health and disease has been intensively investigated particularly in the past three decades, but still involvement of PKCα in multiple biological processes is unclear. Genetically modified mice are commonly used to analyze gene and protein functions within the organism aiming at a precise translation onto human conditions. Among mouse models, knockout mice enable total gene ablation and entire functional gene characterizations.
In the present work, PKCα-deficient mice were characterized in order to determine the functional role of PKCα in smooth muscle cell contraction particularly in the pulmonary vascular system. Formation of megaesophagus was a random observation in PKCα-deficient mice and was further investigated with focus on smooth muscle cell distribution in the lower esophageal sphincter.

1.1 Protein Kinase C (PKC) and its Role in Smooth Muscle Contraction

1.1.1 Overview of the PKC Family

Protein Kinase C is a family of serine/threonine kinases, affecting a wide range of intracellular signal transduction pathways. Multiple studies outline its substantial relevance to pathological conditions, including heart failure, pain and diabetes (Mochly-Rosen et al. 2012). Most of the PKC family members are ubiquitously expressed, but tissue-specific expression has also been reported. All PKC family members consist of a highly conserved catalytic and a regulatory domain, the latter ensuring that the enzyme remains in an inactive status. PKC classification into conventional, novel and atypical PKC isozymes (cPKC, nPKC, aPKC, respectively) correlates with their regulatory domain structure and consequent way of activation (Steinberg 2008).
Initial descriptions on PKC activity were published in 1977 by Nishizukas´ working group (Takai et al. 1977). Following studies identified tumor promoting phorbol ester and the second messenger diacylglycerol (DAG), a natural degradation product of phosphatidylinositol, as PKC activators (Castagna et al. 1982, Takai 2012). While conventional PKC isozymes (PKCα, PKCβ_I, PKCβ_{II}, PKCγ) and novel PKCs (PKCδ, PKCε, PKCθ, PKCη, PKCμ) respond to DAG and phorbol ester, only cPKCs additionally require calcium for activation. In contrast, function of atypical PKCs (PKCζ, PKCι/PKCλ) mostly depends on protein-protein interaction.

PKC activation is indicated by translocation from the cytosolic fraction to the plasma membrane, which was first described in 1982 by Kraft and colleagues (Kraft et al. 1982). The classical model of activation by translocation has been demonstrated in various studies for the conventional PKCα isozyme (Ng et al. 1999, Wagner et al. 2000).

PKC isozymes are widely expressed in all tissues and PKCα has been shown to play a crucial role in numerous cellular processes including proliferation, differentiation, migration, adhesion and apoptosis (Nakashima 2002). PKC sensitivity to tumor promoting phorbol ester led to investigations on the "PKCα isozyme" and its role in cancer, but PKCα has also been intensively discussed in the context of cardiovascular diseases and platelet function (Konopatskaya and Poole 2010).

1.1.2 PKC in Smooth Muscle Contraction

Increasing intracellular Ca^{2+} concentration is the central trigger for smooth muscle contraction. Ca^{2+} influx allows formation of the calcium-calmodulin complex (Ca^{2+}-CaM), which leads to myosin light chain kinase (MLCK) activation followed by myosin light chain (MLC) phosphorylation at amino acid serine 19. Subsequently, actin evokes myosin ATPase activation and cross-bridge cycling of actin and myosin filaments can occur. This actin-myosin interaction appears as smooth muscle contraction (Somlyo and Somlyo 1994).

According to tissue-specific smooth muscle cell (SMC) distribution, different organ systems display distinct smooth muscle cell functions termed phasic (fast-rhythmic) or tonic (slow-sustained) contractile function. In the vascular system, vascular smooth muscle cell (VSMC) contraction ensures high-pressure systemic and low-pressure pulmonary circulation. Particularly in arteries smooth muscle cells are the most represented cell type. Distinctive for VSMCs is the fact that tonic contractile function is present in large arteries and veins, whereas phasic contractile function is characteristic for smaller arteries (Reho et al. 2014).

Most Ca^{2+} entry and therefore contractile mechanisms in VSMCs are directly regulated through voltage-operated calcium channels (VOC), receptor-operated calcium channels (ROC) and store-operated calcium entry mechanisms (SOC). VOC activation is primarily dependent on the membrane potential, meaning that membrane depolarization by firing of action potentials causes VOC opening. Receptor-operated Ca^{2+} influx is mediated through G protein-coupled receptor-ligand binding and phospholipase C (PLC) activation, which subsequently provokes ion channel stimulation. Store-operated calcium entry mechanisms lead to activation of Ca^{2+} receptors in order to refill intracellular calcium stores due to decreased Ca^{2+} storage in the sarcoplasmatic reticulum (Goulopoulou and Webb 2014). In contrast, indirect calcium influx occurs as a consequence of K^+ channel inhibition (Olschewski et al. 2014). Both direct and indirect calcium entry mechanisms involve

activation of PKC via DAG, phorbol ester or Ca^{2+}. Subsequently, PKC either directly mediates MLC phosphorylation or leads to the activation of further signaling proteins such as C-kinase-activated protein phosphatase-1 inhibitor (CPI-17kDa) eventually resulting in smooth muscle contraction (Figure 1). In VSMCs, VOCs are mostly represented by L-type (long-lasting) voltage-gated Ca^{2+} channels, which have been known to be stimulated in a PKC-dependent manner (Schuhmann and Groschner 1994) and PKCα seems to be associated with L-type VOC activation in arterial smooth muscle cells (Santana et al. 2008). Likewise, SOCs studied in portal vein myocytes were linked to PKC-mediated signal transduction pathways (Albert and Large 2002). ROC activation via contractile agonists such as thromboxane A2, endothelin-1, serotonin, angiotensin II or prostaglandin F2α provokes PKC-dependent vascular smooth muscle contraction including conventional, novel and atypical PKC isozymes (Barman and Pauly 1995, Barman et al. 1997, Kanashiro et al. 2000, De Witt et al. 2001). Involvement of PKCα in receptor agonist-induced vasoconstriction has been reported for the ferret aorta (Lee et al. 1999), rat mesenteric arteries (Ohanian et al. 1996) as well as porcine and human coronary arteries (Dallas and Khalil 2003, Feng et al. 2010).

Among K^+ channels, voltage-gated K^+ channels (K_V) display the largest and best characterized group in VSMCs. K_V channel inhibition and pulmonary vascular contraction have been shown to be associated with activation of atypical PKCζ (Cogolludo et al. 2003), whereas blockage of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) involves conventional PKC isozymes (Schubert et al. 1999). Other families of K^+ channels have also been reported in agonist-induced vasoconstriction via G protein-coupled receptor stimulation and subsequent activation of PKC isozymes. Investigations on TASK-1, a member of the KCNK family (also known as KCNK3), provided evidence that endothelin-1 induces vasoconstriction via TASK-1 inhibition (Tang et al. 2009). ATP-sensitive K^+ channels were shown to be inhibited via angiotensin II signaling in a PKCε-dependent manner (Hayabuchi et al. 2001).

Furthermore, PKC isozymes have been identified as relevant contractile modulators in smooth muscle cells of the gastrointestinal tract. Here, phasic smooth muscle cell contraction plays a crucial role, mainly in peristaltic movements. On the contrary, adequate sphincter function is ensured through sustained smooth muscle cell contraction. Esophageal motility and in particular lower esophageal sphincter function has been investigated in several studies on the feline esophagus (Sohn et al. 2001, Cao et al. 2003, Kim 2004, Harnett et al. 2005). Sohn and co-workers provided evidence for the $PKC\beta_{II}$ isozyme to mainly regulate sustained contraction and thereby maintenance of the lower esophageal sphincter tone (Sohn et al. 1997). Moreover, sustained intestinal smooth muscle cell contraction was shown

to involve PKCα activity in experiments with intestinal tissue from guinea pigs (Murthy et al. 2000).

Based on their substantial role in regulatory processes of smooth muscle cell function, PKC isozymes and particularly PKCα represent potential therapeutic targets in smooth muscle disorders and vascular diseases as pulmonary hypertension.

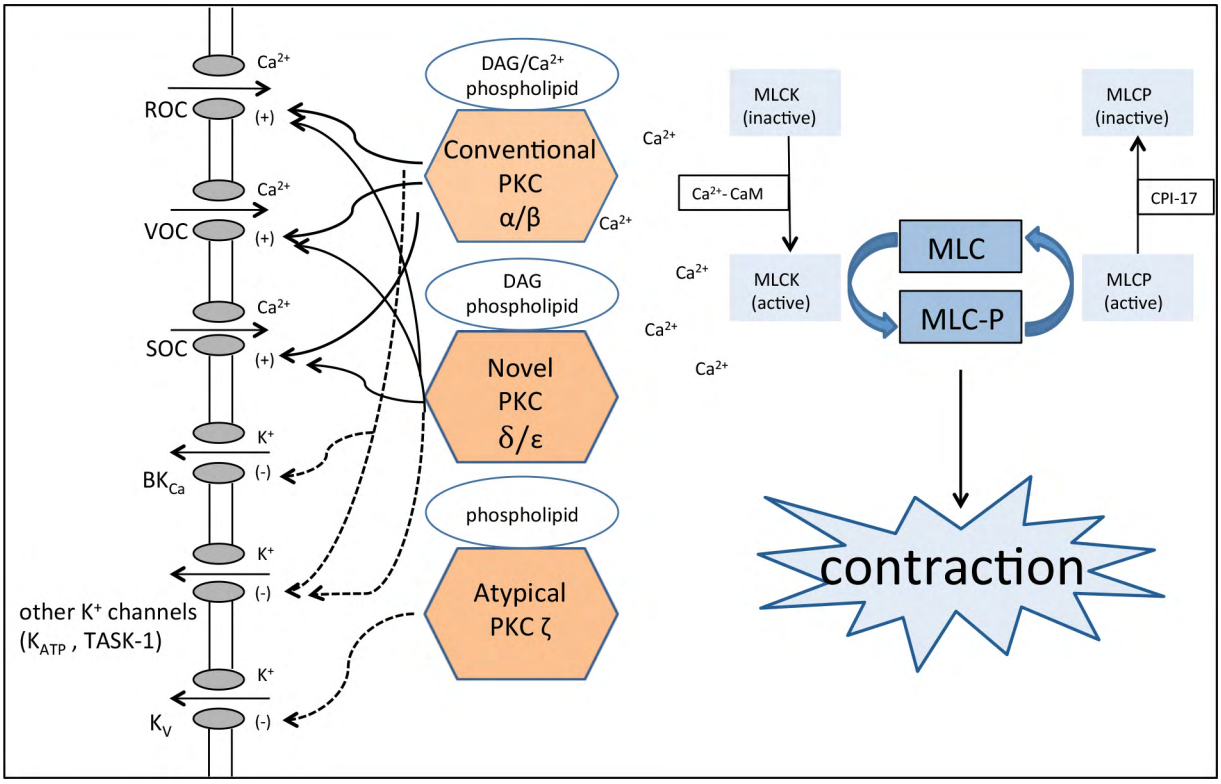

Figure 1: Protein kinase C isoforms in vascular smooth muscle contraction modified after (Ward et al. 2004)

Solid lines refer to stimulation, dashed lines to inhibition. Abbreviations are as follows: BK_{Ca}, large conductance Ca^{2+}-activated K^+ channels; CaM, calmodulin; CPI-17, C-Kinase-activated Protein phosphatase-1 Inhibitor, 17kDa; DAG, diacylglycerol; K_{ATP} , ATP-sensitive K^+ channels; K_V, voltage-gated K^+ channels; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ROC, receptor-operated Ca^{2+} channels; SOC, store-operated Ca^{2+} channels; VOC, voltage-operated Ca^{2+} channels.

1.2 Pulmonary Arterial Pathology in Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a rare but fatal condition characterized by pathological modifications of precapillary pulmonary arteries. In PAH patients, the pulmonary arterial pressure exceeds 25 mmHg (3.33 kPa) under rest. Endothelial dysfunction, enhanced vasoconstriction and pulmonary arterial remodeling including *in situ* thrombosis contribute to a progressive increase in pulmonary vascular resistance. The updated official pulmonary hypertension (PH) classification compiled at the 4th World Symposium on PH in Dana Point, California in 2008 and reviewed at the 5th World Symposium in 2013 in Nice, France, declares PAH as group one out of five different PH forms and subdivides PAH into idiopathic, heritable, drug- and toxin-induced or associated with other disorders (Simonneau et al. 2009, Simonneau et al. 2013). The estimated PAH prevalence in humans ranges from 15-26 cases per million (Humbert et al. 2006, Peacock et al. 2007, Jansa et al. 2014). Left untreated, this progressive disease causes right heart failure with lethal outcome.

1.2.1 Pathogenesis

The arterial vessel wall is built of the tunica intima, tunica media and tunica adventitia. In PAH, progressive remodeling processes are observed in all three tissue layers. Adventitial fibroblasts seem to proliferate early, are able to differentiate into myofibroblasts and evidently migrate towards the vascular lumen (Stenmark et al. 2013). Medial thickening appears as a consequence of smooth muscle cell hypertrophy and hyperplasia. Further, *de novo* muscularization of previously non-muscularized pulmonary arteries is a common histopathological finding (Stacher et al. 2012). At advanced disease stages, focal endothelial proliferation together with medial and adventitial thickening cause formation of characteristic plexiform lesions, which may expand into perivascular areas (Pietra et al. 2004). Pathomechanisms contributing to these vascular lesions are complex and inflammation is discussed as a primary stimulus (Price et al. 2012, Kherbeck et al. 2013). Moreover, upregulated secretion of matrix metalloproteinases has been shown to play a crucial role in proliferation and migration processes of endothelial cells (EC), SMCs and fibroblasts (Chen et al. 2013). Ultimately, these cellular changes result in severe pulmonary arterial remodeling.

Besides the structural alterations, pulmonary arterial function is strongly impaired due to altered molecular mechanisms. An imbalance between vasoconstrictors and vasodilators causes an increased response to vasoconstrictive stimuli and results in the PAH-characteristic hypertensive state.

Elevated plasma levels of the potent vasoconstrictor endothelin-1 (ET-1) and altered expression of ET-1 receptors are distinctive in PAH patients highlighting the key role of ET-1 in the pathogenesis of PAH (Giaid et al. 1993, Davie et al. 2002). ET-1 is mainly produced by ECs and signals via G protein-coupled ET-1 receptors type A (ET_A) and type B (ET_B). Vascular smooth muscle cell contraction is mediated in a PKC-dependent manner through ET_A and partly ET_B activation. In contrast to ET_A, ET_B expression on VSMCs is sparse, while ET_B is mainly expressed on ECs. ET_B stimulation on ECs evokes the release of vasodilating mediators such as nitric oxide (NO) and prostacycline (PGI2). Hence, ET-1-induced vasoconstriction mainly involves ET_A whereas vasodilation is promoted by ET_B. Cytokines, growth factors, hypoxia and shear stress have been described to induce ET-1 generation, which in turn promotes smooth muscle cell proliferation, migration, inhibition of apoptosis and endothelial tubule formation (Shao et al. 2011). Interestingly, increased ET_B expression was demonstrated in idiopathic PAH patients (Bauer et al. 2002) whereas decreased ET_A/ET_B expression has been described for heritable PAH (Talati et al. 2010).

Besides ET-1, circulating serotonin (5-hydroxytryptamin, 5-HT) levels are found to be elevated in PAH patients (Herve et al. 1995). Plasma serotonin derives from enterochromaffin cells and is stored in platelets. However, in PH patients, serotonin was shown to be produced by pulmonary arterial endothelial cells (Eddahibi et al. 2006). The serotonin transporter (5-HTT) as well as 3 out of 14 known 5-HT receptors, all of them located on the smooth muscle cell membrane, have been identified to be involved in PAH pathogenesis. 5-HT_{2A}, 5-HT_{2B} and 5-HT_{1B} receptor stimulation mediates pulmonary vasoconstriction, and overexpression of 5-HT_{1B} has been reported for experimentally induced PAH (Rondelet et al. 2003). Moreover, mice overexpressing 5-HTT have been shown to be highly susceptible to hypoxia-induced PAH verifying the correlation between the mitogenic impact of 5-HT and 5-HTT action (MacLean et al. 2004). This 5-HTT-evoked effect on pulmonary arterial smooth muscle cells was suggested to be due to a crosstalk between 5-HTT and 5-HT_{1B} (Morecroft et al. 2005).

Investigations on prostacyclin and its opponent thromboxane A2 (TXA2) further demonstrate the imbalance between vasodilators and vasoconstrictors in PAH. Physiologically, prostacyclin is synthesized in endothelial cells and inhibits SMC contraction through intracellular cyclic AMP increase, whereas thromboxane A2 causes PKC-involved pulmonary SMC contraction. Both, prostacyclin and thromboxane A2 signal via specific G protein-coupled receptors. Christman and colleagues measured urinary excretion of stable TXA2 and PGI2 metabolites from idiopathic PAH patients and found increased TXA2 but decreased PGI2 metabolite levels (Christman et al. 1992). Elevated TXA2 levels were suggested to result from an increased interaction between platelets and pulmonary arterial

walls in PAH patients (Lannan et al. 2014). Furthermore, decreased levels of PGI2 synthase were detected in patients with severe PH (Tuder et al. 1999).
Dysregulation of ion channels, in particular Kv channels, is another characteristic of PAH and vasoconstrictive agents, including TXA2 and ET-1, which are both increased in PAH patients, reduce Kv currents. In patients with idiopathic PAH, altered expression and function of the Kv1.5 channel was shown. This decreased Kv1.5 expression was linked to various single nucleotide polymorphisms in the Kv1.5 gene *KCNA5* (Remillard et al. 2007).
The pathobiology of PAH is multifactorial comprising further signaling cascades including the involvement of vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-ß) and the angiogenic factor angiopoietin 1 (Humbert et al. 2004). Moreover, mutation in the bone morphogenetic receptor 2 (BMPR2) gene is a frequent phenomenon associated with heritable PAH (Deng et al. 2000).
The PAH dominating enhanced vascular resistance is, as mentioned before, reflected by hyperresponsiveness. Most common PAH studies with rodents use the monocrotaline, T-helper (Th)2 inflammation or hypoxia model to induce and investigate hyperresponsiveness. Nevertheless, the exact pathomechanisms mediating PAH remain unclear.

1.2.2 Treatment of Pulmonary Arterial Hypertension

A better understanding of PAH pathogenesis in the last two decades markedly improved treatment success but PAH is still a progressive and life threatening disease with poor prognosis and high morbidity.
There are distinct therapeutic approaches, all of them focusing on vasodilation and delay of pulmonary arterial remodeling (Seferian and Simonneau 2013).
The potent vasodilating, antiproliferative and antithrombotic effects of prostacyclin are utilized with different prostacyclin analogues, which are administered subcutaneously, intravenously or orally. Some of the systemic side effects can be prevented by inhalation, a better tolerated way of administration.
Selective and non-selective ET-1 receptor antagonists (ERA) are available for PAH treatment and achieve vasorelaxation through ET_A or combined ET_A and ET_B antagonisms on VSCMs. However, risk of hepatic injury requires continuous monitoring of patients receiving ERAs.
Treatment with phosphodiesterase type 5 (PDE-5) inhibitors exerts protective vasodilatory effects through modulation of NO signaling. Physiologically, NO induces vasodilation via intracellular production of cyclic guanosinmonophosphate (cGMP) in smooth muscle cells, whereas PDE-5 degrades cGMP and thus promotes vasoconstriction. Hence, PDE-5 inhibitors evoke an increase in cGMP, which blocks Ca^{2+} influx and subsequently induces relaxation of the smooth muscle cell.

Approximately 10% of PAH patients show acute pulmonary vasoreactivity. In these patients, administration of high dosed calcium channel blockers confers considerable clinical improvement.

More recently, a stimulator of soluble guanylate cyclase (sGC) has been approved for PAH treatment as a novel therapeutic approach. Activation of sGC with orally administered Riociguat increases sGC sensitivity to NO, thereby evoking increased cGMP synthesis and subsequent vasodilation.

Though PAH remains incurable, current pharmacological therapies help to manage and delay disease progression. A US study from 2012 compared estimated PAH survival rates from the PAH registry of the National Institutes of Health (NIH) introduced in the 1980s, to results assessed from the newly established „Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management" (REVEAL) including 2635 patients enrolled from 2006-2009. In contrast to median survival of 2.8 years given by the NIH registry, data analysis from the REVEAL registry estimated a median survival time of more than 7 years for PAH patients (Benza et al. 2012). This improvement is a result of the progress made in PAH research.

1.2.3 Animal Models in PAH Research

The first systematic studies on pulmonary hypertension in animals and humans emerged with the discovery of hypoxic pulmonary vasoconstriction (HPV) by von Euler and Liljestrand in 1946 (von Euler and Liljestrand 1946). Briefly, HPV means constriction of pulmonary arteries in the presence of low oxygen levels redirecting capillary perfusion towards better-oxygenated alveoli. This mechanism of local blood flow regulation prevents inadequate blood oxygenation by adapting pulmonary perfusion to ventilation (Sylvester et al. 2012).

Experimentally induced HPV in mechanistic studies has been shown to be biphasic. Acute HPV includes a peak ~600 seconds (s) post induction and after reaching a plateau sustained HPV follows (Wiener and Sylvester 1991, Weissmann et al. 1995, Connolly et al. 2013). While intrinsic signal transduction mechanisms of hypoxia sensing have been described for isolated PASMCs and pulmonary artery rings, HPV in the intact lung involves hypoxia sensing at the alveolo-capillary level, retrograde signal transduction to upstream pulmonary arterioles and subsequent contraction of PASMCs (Sylvester et al. 2012, Wang et al. 2012).

Chronic hypoxia models have been used to investigate PH and PAH pathomechanisms in numerous studies with high predictability and reproducible properties within a selected animal strain. Structural changes mimicking vascular remodeling, one of the hallmarks of PAH, right ventricular hypertrophy and inflammatory infiltration are distinctive for chronic

hypoxia exposure (Stenmark et al. 2009). However, there are species-specific differences regarding the severity of signs. Due to a higher susceptibility compared to mice, rats became the preferred experimental animal among rodents. Nevertheless, hypoxia-induced vascular remodeling observed in neonatal calves has been shown to be more severe than in mice or rats (Stenmark et al. 1987, Stenmark et al. 2009).

The limitations of the hypoxia model have been extensively discussed, mainly criticizing the lack of intimal proliferation as typically seen in PAH patients. Indeed, in rats a combination of hypoxia with the VEGF receptor blocker Sugen-5416 has been observed to cause endothelial proliferation and irreversible vascular remodeling, therefore better resembling PAH (Taraseviciene-Stewart et al. 2001).

Monocrotaline is a toxic pyrrolizidine alkaloid that has been used in PAH modeling for decades. Because of their limited ability to metabolize monocrotaline to its active form, mice are not suitable for the well-established single-hit setting. In contrast, a single injection of monocrotaline evokes severe PH in rats (Gomez-Arroyo et al. 2012). The exact underlying mechanisms of monocrotaline-induced pulmonary damage characterized by vascular remodeling are still unknown but direct endothelial injury is assumed to be the most important trigger. Indeed the monocrotaline model rather matches an acute toxic condition and, unlike the human PAH, has been shown to be preventable and even curable by various agents (Stenmark et al. 2009). Nevertheless a combination of monocrotaline administration and pneumectomy achieved irreversible intimal remodeling through hemodynamic changes (Tanaka et al. 1996, Okada et al. 1997).

More recently, special emphasis was placed on inflammation and its impact on PAH pathogenesis. As a consequence new PAH models investigating Th2-induced immune response to repeated antigen exposure such as *Aspergillus fumigatus*, ovalbumin and house dust mite got established (Tormanen et al. 2005, Witzenrath et al. 2006, Daley et al. 2008, Mushaben et al. 2012). Though it is an ongoing discussion whether inflammation is a cause or consequence of PAH, Th2 models have been described to best exemplify the complexity of vascular remodeling by promoting severe wall thickening of small and medium caliber arteries (Gomez-Arroyo et al. 2012).

Development of genetically modified animals enabled deeper insights into PAH pathogenesis. Particularly the role of *BMPR2* signaling in PAH attempted various investigations on *BMPR2* mutations in mouse models ranging from heterozygous deficiency to tissue-specific knockdowns (Beppu et al. 2004, Hong et al. 2008, West et al. 2008). Though these studies provided varying results regarding PAH resembling signs they serve as crucial resources for ongoing research.

Use of mutant mice in PAH models has also been demonstrated with studies on IL-6 overexpression and deficiency under hypoxic conditions. In PAH patients, elevated plasma

levels of IL-6 were shown to negatively correlate with survival (Itoh et al. 2006, Soon et al. 2010). Moreover, murine IL-6 overexpression caused angioproliferative and inflammatory changes while IL-6 deficiency decreased medial thickening of muscular pulmonary vessels in hypoxia-exposed mice (Savale et al. 2009, Steiner et al. 2009).

In summary, investigating PAH by means of genetic modifications resulted in a better understanding of the disease but there is still much to learn from animal models regarding PAH pathogenesis and mechanistic-functional consequences.

1.3 Megaesophagus Development in Human Achalasia

Human achalasia is defined as esophageal aperistalsis and impaired relaxation of the lower esophageal sphincter (LES). Failure of adequate food bolus passage leads to esophageal dilatation and may result in formation of megaesophagus.

1.3.1 Pathogenesis and Predisposing Factors

The hallmark of achalasia is degeneration of inhibitory nitrinergic neurons from the esophageal myenteric plexus. Consequently, decreased release of relaxant mediators, particularly NO, leads to an imbalanced interaction between the neuronal and muscle cells, resulting in failure of smooth muscle relaxation and motility dysfunction of the striated muscle (Ghoshal et al. 2012).

Idiopathic achalasia is the most common form of achalasia with an estimated incidence of 1.6 per 100.000 per year (O'Neill et al. 2013). It usually occurs between 21 and 60 years with an average age above 50 (Farrokhi and Vaezi 2007, Boeckxstaens et al. 2013). Familial achalasia diagnosed in children is often associated with other complex syndromes such as Down and Allgrove syndrome (Gockel et al. 2010, Torab et al. 2012).

Injury by viral infection is discussed as the initial stimulus contributing to idiopathic achalasia. Subsequently, inflammatory processes provoke destruction of the myenteric plexus and cause loss of nerve fibers and ganglia. In the later stages of the disease, replacement of myenteric nerves is histologically seen as fibrosis (Goldblum et al. 1996, Raymond et al. 1999, Clark et al. 2000, Villanacci et al. 2010). Several viral agents, most notably *herpes simplex*, human papilloma and measles virus have been considered to be involved in the achalasia priming injury but a clear causal relationship is missing.

In patients with achalasia, lack of neuronal NO synthase (nNOS) activity, the major source of NO inside the LES, and a decreased number of NO containing neurons have been reported (Mearin et al. 1993, De Giorgio et al. 1999, Hoshino et al. 2013). A study with nNOS-deficient mice (*nNOS*$^{-/-}$) showed LES hypertension and impaired LES relaxation (Sivarao et al. 2001). This constant failure of proper LES relaxation causes esophageal congestion and may lead to dilatation of the esophageal body (EB).

In the last decade, various mutant mouse models provided increasing evidence for genetic predisposition in the pathogenesis of achalasia. For instance in Allgrove syndrome, mutations in the *AAAS* gene (achalasia, adrenocortical insufficiency, alacrimia syndrome) contribute to achalasia (Tullio-Pelet et al. 2000). However, latest achalasia research has focused more on genes that directly trigger inflammation and esophageal motor dysfunction (Gockel et al. 2010). A candidate gene for inflammatory processes has been outlined by van

der Weyden and co-workers, who observed spontaneous development of megaesophagus associated with chronic inflammatory infiltration and a reduced number of nerves in the myenteric plexus and esophageal muscle layers of *Rassf1a*-null mice (van der Weyden et al. 2009). Moreover, numerous genes presumably modulating esophageal motor function have been determined. Analysis of *Isc/p115*-deficient mice revealed an achalasia like phenotype characterized by esophageal motor dysfunction and progressive dilation (Zizer et al. 2010). Loss of the *Sprouty2* gene has been shown to cause hypersensitivity to glia cell line-derived neutrophic factor (GDNF), resulting in enteric nerve hyperplasia and subsequent esophageal achalasia (Taketomi et al. 2005). Moreover, formation of megaesophagus was seen in α adducin (*Add1*)-deficient mice (Robledo et al. 2012). Apart from infectious agents and genetic predisposition, autoimmune conditions have been discussed in the pathogenesis of achalasia. Booy and colleagues reported a 3.6 fold increased likelihood for achalasia patients to suffer from autoimmune disease compared to healthy controls (Booy et al. 2012).
It is obvious that achalasia etiology is multifactorial and requires more research.

1.3.2 Treatment

Surgical intervention is the method of choice in achalasia treatment (Francis and Katzka 2010, Patti and Fisichella 2014). Pneumatic balloon dilatation enables sustained distention of the LES by inserting and inflating a balloon positioned at the esophagogastric junction. The more frequently used laparoscopic Heller myotomy, named after the German surgeon Ernst Heller, requires myotomy of distal esophageal muscle layers and the LES. Less invasive endoscopic esophagomyotomy techniques have been established and performed more recently including surgical variations of the classical Heller myotomy (Boeckxstaens et al. 2013). For both methods, pneumatic balloon dilatation and myotomy, a similar treatment success of ~90% has been reported in a comprehensive and comparative study by Boeckxstaens and colleagues (Boeckxstaens et al. 2011).
Pharmacological therapy with long-acting calcium channel blockers and dephosphorylation of myosin light chain with nitrates achieves limited benefit in patients who are not suitable for surgical intervention (Hoogerwerf and Pasricha 2001). Decent but only temporary therapeutic success can be reached via injection of botulinum toxin into the LES. Subsequently blockage of acetylcholine release from the nerve endings evokes LES relaxation (Pasricha et al. 1995). Alternatively, insertion of removable metallic stents may serve as an effective long-term treatment (Zhao et al. 2009).

1.4 Hypotheses and Aims of the Study

It has been shown that PKC mediates smooth muscle cell contraction. However, the effects of PKCα deficiency on pulmonary vascular responsiveness are unknown. Thus, the present study characterized PKCα deficiency in homozygous knockout mice.
Due to the random finding of megaesophagus in mice lacking PKCα, the influence of PKCα deficiency was further investigated with regard to smooth muscle cells of the esophagus.

The following hypotheses were tested:

1. PKCα modulates pulmonary vascular function.

2. PKCα influences smooth muscle cell morphology of the lower esophageal sphincter (LES), and loss of PKCα evokes changes in the LES which contribute to formation of megaesophagus.

2 Animals, IPML Technique and Study Design

2.1 Animals

PKCα$^{-/-}$ mice were initially generated on a 129 SV background by Leitges and colleagues who did not observe any abnormalities regarding viability, fertility and external characteristics (Leitges et al. 2002). Our own colony of *PKCα*$^{-/-}$ mice was bred at the research institute for experimental medicine (Charité Universitätsmedizin Berlin, Germany) and corresponding 129 SV wildtype mice were purchased from Charles River (Sulzfeld, Germany). *PKCα*$^{-/-}$ and WT mice at the age of 8-11 weeks and 14 months were used for all experiments. Mice were housed under specific pathogen-free conditions in individually ventilated cages within a climate- and light cycle-controlled animal house and fed standard chow and tap water ad libitum.

Data from 150 mice are included in study 3.1 “PKC alpha deficiency in mice is associated with pulmonary vascular hyperresponsiveness to thromboxane A2 and increased thromboxane receptor expression”. Study 3.2 “Juvenile megaesophagus in *PKCα*$^{-/-}$ mice is associated with an increase in the segment of the distal esophagus lined by smooth muscle cells” contains data from 86 mice of which most have been used also for experimental procedures of study 3.1. In total, 182 mice were used for both studies.

2.2 Isolated Perfused and Ventilated Mouse Lung

The isolated perfused and ventilated mouse lung (IPML) is an *ex vivo* technique which enables analyses of pulmonary vascular and airway functions (Figure 2).

This experimental set-up simulates physiological conditions and thereby allows to investigate pulmonary responsiveness in the undamaged lung similar to mechanisms in the intact organism.

Mice were anesthetized via intraperitoneal injection with 16×10^{-4} kg Ketamin and 75×10^{-5} kg Xylazin per kg of bodyweight. For surgical procedures, mice were placed into a water-jacketed plexi glass chamber. Tracheal intubation allowed positive pressure ventilation in a 90 breaths × min^{-1} frequency. Ventilation gas contained 5% CO_2, 20% O_2 and 75% N_2. After laparotomy, perforation of the diaphragm was performed for intracardial injection of 125 I.U. heparin sodium thereby preventing thrombus formation. *Vena cava* exsanguination followed and the abdomen was completely removed. Preparation of the lung and heart required thoracotomy and removal of the thymus and pericard. The pulmonary artery was cannulated with a purpose-built stainless steel catheter in order to flush the lung

vasculature with heated (37°C) and carbonated (0.02 mol/L $NaHCO_3$) Krebs-Henseleit-hydroxyethylamylopectin buffer. Subsequently, pulmonary arterial pressure measurement was performed. A roller pump kept the perfusion at a constant flow of 1 mL × min^{-1} in a nonrecirculating system. A second cannula was placed into the opened up left atrium to allow venous perfusate outflow, pressure measurement and adjustment. With a pressure equilibration vessel the left atrial pressure was set at +2.2 cmH_2O (0.22 kPa).

The chamber was closed and negative pressure ventilation was generated through a venturi gauge. End-expiratory and inspiratory pressure was set at -4.5 and -9.0 cmH_2O (-0.44 and -0.88 kPa), respectively. A pneumotachometer measured velocity of airflow. Hyperinflation (-24 cmH_2O (-2.35 kPa)) at 240 s intervals prevented atelectasis. All data were measured with differential pressure transducers, amplified and recorded with pulmodyn software. Hardware and software were purchased from Hugo Sachs Elektronik (HSE)-Harvard Apparatus.

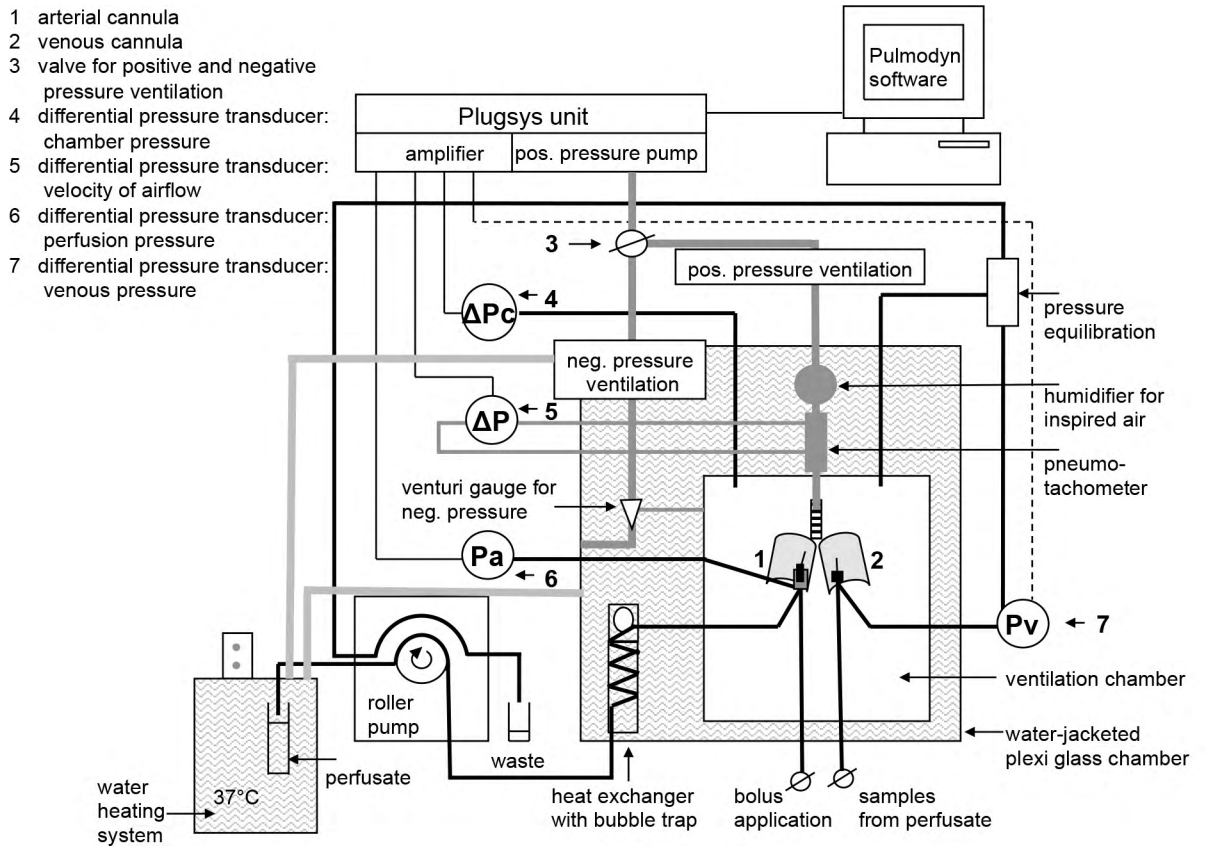

Figure 2: General arrangement of the isolated perfused and ventilated mouse lung modified after (von Bethmann et al. 1998).

2.3 Study Design

For the first time, isolated perfused lungs of *PKCα*$^{-/-}$ mice were studied. In PAH, an increased pulmonary vascular responsiveness to endogenous vasoconstrictive mediators such as ET-1, TXA2 and serotonin is believed to be a consequence of changes in pulmonary arterial smooth muscle cell function and morphology.

Here, pulmonary vascular responsiveness towards vasoconstrictive stimuli was investigated taking two approaches. On the one hand examination of genetic PKCα deficiency and on the other hand pharmacological PKC inhibition with the broad spectrum PKC inhibitor bisindolylmaleimide I (BIM) and the selective PKC inhibitor Gö6976 of conventional PKC isozymes PKCα and PKCβ was conducted to gain deeper insights into the PKC family as vasoactive modulators in the lung. Moreover selective PKCı inhibitor sodium aurothiomalate hydrate (ATM) was used to further investigate TXA2-induced vasopressor response.

Increasing concentrations of ET-1, serotonin or TXA2 analog U46619 were administered into the perfusion buffer, applied for 600 (ET-1), 30 (serotonin) or 180 s (U46619), respectively and the pulmonary vascular responsiveness in *PKCα*$^{-/-}$ and corresponding WT mice was recorded. For analysis of alveolar hypoxia, mean pulmonary arterial pressure was recorded for 12 min under hypoxic ventilation (1% O_2) in a recirculating perfusion system. Interaction between the volumetric flow-rate (Q) and the vascular pressure (P) was recorded prior to and 600 s after induction of HPV. For this purpose the basal perfusion rate of 1 mL × min^{-1} was changed to 0.5, back to 1, to 1.5 and to 2 mL × min^{-1} for 30 s each. This four-point pressure-flow (P-Q) curve was performed to test distensibility of the pulmonary vasculature under normoxic and hypoxic conditions. Endogenous effects of NO were prevented by Nω-Nitro-L-arginin-methylester-hydrochlorid and the impact of PGI2 by addition of indomethacin to the perfusion buffer. Pulmonary arterial baseline pressure was recorded 30 seconds prior to vasoconstrictive stimulation. BIM (1 × 10^{-6} mol/L), Gö6976 (1 × 10^{-6} mol/L), ATM (2 × 10^{-5} mol/L) or solvent (<1% DMSO) was added to the perfusion buffer. BIM and Gö6976 were dissolved in DMSO while ATM was dissolved in perfusion buffer.

For mRNA analysis from microdissected intrapulmonary arteries laser capture microdissection (LCM) was performed. This microscopy method allows laser-assisted precise collection of tissue samples for sensitive downstream analyses. Here, LCM enabled careful picking of intrapulmonary arteries.

Investigations on the esophagus with regard to the lower esophageal sphincter were conducted with 9-10-week- and 14-month-old mice. The whole esophagus was dissected for esophageal measurements, histological and mRNA expression analyses.

3 Own Research Publications

All results discussed in the current work are part of two research publications (3.1 and 3.2). The table attached in the appendix contains numeric data of study 3.1 “PKC alpha deficiency in mice is associated with pulmonary vascular hyperresponsiveness to thromboxane A2 and increased thromboxane receptor expression”. These data are listed as median, minimum and maximum values.

3.1 PKC alpha deficiency in mice is associated with pulmonary vascular hyperresponsiveness to thromboxane A2 and increased thromboxane receptor expression

“PKC alpha deficiency in mice is associated with pulmonary vascular hyperresponsiveness to thromboxane A2 and increased thromboxane receptor expression”

Authors: Christoph Tabeling*, Elena Noe*, Jan Naujoks, Jan-Moritz Doehn, Stefan Hippenstiel, Bastian Opitz, Norbert Suttorp, Robert Klopfleisch, Martin Witzenrath

* these authors contributed equally to this work

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Declaration of own portion of work in this research publication:

Contributions by E. Noe: Design, preparation, completion and evaluation of experiments as well as subsequent preparation of the manuscript.

Contributions of other authors: Design of experiments, data interpretation, preparation and editing of manuscript.

3.2 Juvenile megaesophagus in *PKCα*$^{-/-}$ mice is associated with an increase in the segment of the distal esophagus lined by smooth muscle cells

"Juvenile megaesophagus in *PKCα*$^{-/-}$ mice is associated with an increase in the segment of the distal esophagus lined by smooth muscle cells"

Authors:	Elena Noe, Christoph Tabeling, Jan Naujoks, Jan-Moritz Doehn, Bastian Opitz, Stefan Hippenstiel, Martin Witzenrath, Robert Klopfleisch
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	Please purchase this part online.

Declaration of own portion of work in this research publication:

Contributions by E. Noe: Design, preparation, completion and evaluation of experiments as well as subsequent preparation of the manuscript.

Contributions of other authors: Design of experiments, data interpretation, preparation and editing of manuscript.

4 Concluding Discussion

In the past 20 years the PKC family has been an attractive target for drug development. However, new issues arise and open questions exist. It is known that activation of PKC isozymes mediates multiple signal transduction pathways and plays a crucial role in the pathogenesis of numerous major diseases. To investigate which isozyme is involved in a certain physiological process or pathological condition, PKC isozyme specific tools are required. Addressing this question, both genetic and pharmacological tools have been used.

Pharmacological PKC inhibition via binding to the isozymes' catalytic or regulatory domain has been demonstrated by different approaches such as competing with ATP, DAG mimicking and inhibition of protein-protein interactions (Mochly-Rosen et al. 2012).

Besides pharmacological protein inhibition, manipulation of the respective gene allows functional protein analysis on another level. Anatomical, physiological and genetical similarities to humans, but particularly advantageous keeping and breeding conditions, promoted the establishment of gene targeting in the house mouse (*mus musculus*). Mutant mouse models range from ubiquitous to tissue-specific insertion, reduction and depletion of gene expressions and thus are attractive resources for defining human gene functions. In constitutive "knockout" mice, the traditional way of gene targeting, a certain gene of interest is permanently inactivated in every cell of the organism (van der Weyden et al. 2002).

All PKC isozymes have been demonstrated to be expressed in the lung and several isozymes including PKCα are known to promote smooth muscle cell contraction (Ward et al. 2004, Dempsey et al. 2007). Hence, the first aim of this study was to determine isozyme selective functions in the pulmonary vasculature. Therefore pulmonary vasopressor response in mice lacking the *PRKCA* gene, encoding for PKCα, were studied in combination with pharmacologic PKC inhibition with ATP-competitive broad spectrum PKC inhibitor BIM and in part with the selective PKCα and PKCβ inhibitor Gö6976 in corresponding wildtype mice. Later, the effect of selective PKCı inhibitor ATM was studied in *PKCα*$^{-/-}$ lungs.

The PKCα knockout mouse was generated and first published by Leitges and colleagues who studied the impact of PKCα on insulin signaling (Leitges et al. 2002).

Since we observed a high prevalence of esophageal dilation in PKCα-deficient mice, the second aim of the study was to further investigate megaesophagus development due to PKCα deficiency with regard to smooth muscle cells of the lower esophageal sphincter and thereby clarify whether and to what extent the absence of PKCα contributes to achalasia-like esophageal malformation.

4.1 PKC in the Murine Pulmonary Vasculature

4.1.1 Hypoxic Pulmonary Vasoconstriction (HPV)

Numerous studies using different species and techniques have been highlighting the involvement of PKC in HPV, but satisfactory results unraveling PKC activation in the complex HPV mechanism are missing. However, Ward and colleagues elegantly reviewed the current knowledge of PKC isozymes in HPV. In this review, a key study was pointed out, in which PKC activation upon HPV was proven by showing an increase in PKC activity towards acute hypoxia in isolated sheep PASMC (Plevin et al. 1994, Ward et al. 2004). Moreover, Weissmann and co-workers were able to demonstrate HPV reduction following nonselective PKC inhibition in isolated perfused rabbit lungs (Weissmann et al. 1999).
In the present study, HPV was unaffected by PKCα deficiency while broad spectrum PKC inhibition with BIM significantly attenuated HPV, suggesting that a PKC isozyme other than PKCα is required for HPV activation. Litter et al. studied acute HPV in isolated perfused mouse lungs of PKC epsilon-deficient mice (*PKCε*$^{-/-}$) and reported a ~50% blunted vasopressor response in *PKCε*$^{-/-}$ compared to *PKCε*$^{+/+}$ mice, while expression of all other PKC isozymes was unaffected by PKCε deficiency (Littler et al. 2003). Tsai and colleagues showed in rat isolated arterial rings that conventional PKC inhibition with Gö6976 had no effect on hypoxia-induced contraction whereas, in line with data from the present study, broad spectrum PKC inhibition markedly reduced HPV (Tsai et al. 2004, Tsai et al. 2007). Taken together these results provide evidence that HPV at least partly requires novel PKC isozyme PKCε, but most likely no conventional PKC isozyme and particularly not PKCα.

4.1.2 Endothelin-1-induced Vascular Responsiveness

Vasoconstriction to ET-1 was markedly reduced following nonselective PKC inhibition with BIM or following conventional PKC isozyme inhibition with Gö6976 and, in contrast to HPV, also in the absence of PKCα. These findings point to a predominant role of PKCα in ET-1-induced vascular smooth muscle contraction and are consistent with results from Feng and colleagues who suggested PKCα signaling in ET-1-induced contractile response of human coronary arterioles by using a specific PKCα inhibitor (Feng et al. 2010). Additionally, reduction of ET-1-induced contraction in isolated mesenteric arteries was seen after treatment with a PKCα inhibitor peptide (Rainbow et al. 2009). The results of the present study provide further evidence for PKCα signaling in ET-1-induced vasopressor response.

4.1.3 Serotonin-induced Vascular Responsiveness

Remarkably, serotonin-induced pulmonary vasoconstriction was not affected by loss of PKCα or PKC inhibition using BIM. Previous investigations assumed that PKC involvement following serotonin stimulation varies species-dependently. Moreover, distinct results were generated using different PKC inhibitors. To make matters more complicated, tyrosine kinase activation has also been shown to be considerably involved in serotonin-induced vascular smooth muscle contraction. The following studies explored effects of serotonin on vasoconstriction and thereby generated, in part, opposing results:

Kaye and co-workers reported that serotonin-induced pulmonary vascular response in the feline was unaffected towards pretreatment with PKC inhibitor staurosporine or calphostin C, while staurosporine diminished the response to serotonin in isolated perfused rat lungs in the same study (Kaye et al. 1995). In another work, serotonin-induced contraction in isolated rat pulmonary arteries showed sensitivity to PKC inhibition by Gö6976 as well as to tyrosine kinase inhibition and further achieved relaxation by treatment with the selective tyrosine kinase inhibitor tryphostin 23 (Cogolludo et al. 2006). Tasaki and co-workers demonstrated a reduction in serotonin-induced contraction after preincubation with the tyrosine kinase inhibitor genistein in rat aortic strips (Tasaki et al. 2003). Investigations of isolated perfused canine lungs by Barman and colleagues provided evidence for the involvement of tyrosine kinase and PKC activity in serotonin-induced vasoconstriction using PKC inhibitor staurosporine, the precursor of BIM with less PKC specificity but higher affinity to other protein kinases (Toullec et al. 1991), and tyrosine kinase inhibitors genistein and tryphostin 23 (Barman et al. 1997). Moreover, the role of tyrosine kinase, particularly the MEK pathway, in serotonin-induced vasoconstriction has been carefully examined in aortic, mesenteric and tail arterial strips from rats (Watts 1996). More recently, Scr-tyrosine kinase-mediated serotonin-induced vasoconstriction in rat mesenteric arterial rings has been reported (Sung et al. 2013).

The present work demonstrates that serotonin-evoked vasoconstriction is not mediated via PKCα in naïve isolated mouse lungs and thereby supports the hypothesis of species-specific differences in serotonin-induced vasopressor response which has been proposed since significant evidence exists for involvement of tyrosine kinase, at least in rat and canine.

Moreover, the current data add to the knowledge that PKC isozyme selectivity profiles of distinct PKC inhibitors vary.

4.1.4 Thromboxane A2-induced Vascular Responsiveness, thromboxane A2 receptor and PKC expression

Vasoconstrictive effects to the thromboxane A2 analog U46619 were only moderately reduced by BIM-evoked broad spectrum PKC inhibition. No effect on U46619-induced vasoconstriction was seen following selective inhibition of conventional PKCα and PKCβ with Gö6976. However, U46619 caused pulmonary vascular hyperresponsiveness in *PKCα*$^{-/-}$ mice. To elucidate this hyperreactivity, mRNA analysis of microdissected intrapulmonary arteries from naïve mice was performed demonstrating that loss of PKCα was associated with an upregulation of thromboxane A2 receptor- and atypical PKCι expression.

Thromboxane A2, a prostanoid derived from arachidonic acid metabolism, induces vascular smooth muscle contraction through activation of the appropriate TP receptor. On vascular smooth muscle cells, TP receptor activation evokes inhibition of Kv channels, subsequent membrane depolarization and activation of L-type Ca^{2+} channels in a PKC-dependent manner. Vasoactive properties of TXA2 have been implicated in the pathogenesis of pulmonary hypertension in a variety of studies. For instance, elevated levels of TXA2 metabolites in PAH patients (Christman et al. 1992) and increased TP receptor density in right ventricle tissue of patients diagnosed with primary PH have been shown (Katugampola and Davenport 2001). So far, no data exist on up- or downregulation of PKCα expression in PAH patients. Experimentally, decreased PKCα activity and expression has been found in pulmonary arteries of rats with chronic hypoxia-induced PH (Shi et al. 2012). Since TP receptor regulation in *PKCα*$^{-/-}$ mice is unclear, compensatory TP upregulation in the constitutive knockout mouse has to be considered as a possible explanation.

Analysis of the PKC expression profile from intrapulmonary arteries revealed upregulation of atypical PKCι mRNA expression in *PKCα*$^{-/-}$ mice, while expression of all other PKC isozymes was unaltered. Upregulation of PKCι and its functional role in the vasculature of *PKCα*$^{-/-}$ lungs was further studied by investigating the effect of PKCι inhibitor ATM on U46619-induced pulmonary vasoconstriction. However, PKCι inhibition did not reduce pulmonary vasopressor response to U46619 in *PKCα*$^{-/-}$ mice. Hence, increased PKCι expression is, other than TP receptor upregulation, most likely not causative for the observed U46619-induced hyperresponsiveness. PKCι has been discussed as a tumor oncogene, but its role in the pulmonary vasculature is currently unknown (Parker et al. 2014). Husain and colleagues examined PKC in rat corpora cavernosa and suggested a potential role for PKCα and PKCι in phenylephrine-induced smooth muscle contraction of the rat corpus cavernosum (Husain et al. 2004). Moreover, it has been perceived that PKCι is activated during embryonic vasculogenesis (Qi et al. 2011). However, previous studies rather outline atypical PKCζ when discussing involvement of PKC in TXA2-induced vasoconstriction. In rats,

Cogolludo et al. demonstrated that U46619-induced vascular smooth muscle contraction via inhibition of voltage gated potassium channels is caused in a PKCζ-dependent manner (Cogolludo et al. 2003). Since the IC_{50} for atypical PKCζ requires BIM 5.8×10^{-6} mol/L, the only moderate reduction in TXA2-induced vasoconstriction by BIM 1×10^{-6} mol/L in the present study seems plausible.

However, Kv inhibition via PKCζ activation has been described to be accompanied by interaction with P62/sequestome1 (Ishii et al. 2013), a scaffolding protein of atypical PKC isozymes, albeit with a higher affinity for PKCι compared to PKCζ (Sanchez et al. 1998).

Consistent with the present observations, earlier studies reported failed inhibition of adrenaline-induced constriction in rat anterior mesenteric artery and vein and epidermal growth factor-urogastrone-evoked contraction in isolated longitudinal smooth muscle strips from guinea-pig stomach by treatment with PKCι inhibitor sodium aurothiomalate (Northover 1967, Itoh et al. 1988). Nevertheless, due to very rare data on selective PKCι inhibition and no other reports on PKCι inhibition in pulmonary vasoconstriction focusing on the specific response to U46619, a potential PKCι-mediated modulation of pulmonary vascular function cannot be entirely ruled out and needs to be further evaluated.

4.2 PKCα Deficiency and Murine Megaesophagus Development

4.2.1 Prevalence of Megaesophagus in *PKCα*$^{-/-}$ and Wildtype Mice

In the present study, megaesophagus was observed with a prevalence of 60% in 9-10-week-old *PKCα*$^{-/-}$ mice compared to 10% in corresponding WT mice of the same age. Esophageal enlargement was also present with a prevalence of 10% in 14-month-old *PKCα*$^{-/-}$ mice and WT mice. To the author's knowledge, there is only one other report on megaesophagus prevalence or incidence in mutant mice (van der Weyden et al. 2009). Van der Weyden and colleagues described a meagesophagus incidence of 20% in *Rassf1a*-null mice compared to 2% in WT littermates, which is similar to the ratio seen in the present study.

4.2.2 Smooth Muscle Cell Distribution

Since megaesophagus in achalasia is a consequence of failed LES function, histological analysis of the distal esophagus including the LES was performed. The main histological finding was that dilated esophagi showed an increased portion of the distal esophagus lined by smooth muscle cells. Anatomically, the esophagus consists of the esophageal body, a tubular muscular structure and the gastroesophageal junction, the LES. In humans, the upper third of the esophageal body is composed of striated muscle while the lower two-thirds as well as the LES are built of smooth muscle cells. In contrast, the mature murine esophageal body fully consists of striated muscle and only the LES is built of smooth muscle cells (Romer et al. 2013). During esophageal maturation, embryonic smooth muscle cells are replaced by striated muscle until postnatal day 14. This process has been investigated in numerous studies suggesting that a subset of smooth muscle cells undergo apoptosis and PKC is well known to be a crucial regulator of apoptosis (Worl and Neuhuber 2005, Rishniw et al. 2007). Since the prevalence of megaesophagus associated with an increased portion of smooth muscle cells was highly increased in young *PKCα*$^{-/-}$ mice, PKCα might be involved in esophageal maturation and loss of PKCα may lead to delayed esophageal maturation.

4.2.3 LES function and PKC Isozyme Expression in the LES

Under physiological conditions the LES remains contracted and thereby prevents gastric reflux. Upon swallowing, LES relaxation allows food bolus passage. This sphincter function is mediated by distinct neuronal triggers. Postganglionic inhibitory neurons modulate LES relaxation via NO release, whereas LES contraction is controlled by preganglionic vagal fibers conducting to the esophageal myenteric plexus (Goyal and Chaudhury 2008). LES

tone maintenance has been demonstrated, at least in feline esophagus, to be dependent on activation of the PKCβII isozyme, which is closely related to PKCα (Sohn et al. 1997, Harnett et al. 2005). However, in the current study, mRNA expression analysis did not reveal a differential PKCß expression in the LES of *PKCα*$^{-/-}$ mice. Notably, decreased mRNA levels of novel PKCδ and PKCε and atypical PKCζ and PKCι in LES of *PKCα*$^{-/-}$ mice were seen. These changes in the isozyme expression profile may contribute to an increased basal LES tonus by so far unknown mechanisms, evoking smooth muscle cell hyperplasia finally resulting in an increased portion of smooth muscle cells in dilated esophagi. Moreover, PKCδ is known to promote smooth muscle cell apoptosis (Ryer et al. 2005) and downregulation of PKCδ in *PKCα*$^{-/-}$ mice possibly prevented proper smooth muscle cell replacement during esophageal maturation.

4.2.4 Age- and Strain-dependent Influence on Megaesophagus Development

The prevalence of megaesophagus in 14-month-old *PKCα*$^{-/-}$ mice was distinctly lower than in 9-10-week-old animals. Thus, megaesophagus seen at young age might not be fatal, but even reversible. Juvenile mice might catch up on esophageal maturation after 9-10 weeks of life. However, since also a few WT mice displayed megaesophagus, there might be so far unknown mechanisms that also contribute to this phenomenon in the 129 SV mouse strain. Nevertheless, loss of PKCα significantly potentiated the chance of megaesophagus formation.

In other mutant mouse models, similar changes were found, supporting the observation that single-gene mutations predispose for esophageal malformation. Loss of the multifunctional cell surface receptor Cdo, collagen19a1 or the *fz4* gene caused enlarged esophagi accompanied by a skeletal-smooth muscle boundary occurring at an abnormally proximal position and impaired esophageal muscle transdifferentiation resulting in the development of megaesophagus (Wang et al. 2001, Sumiyoshi et al. 2004, Romer et al. 2013).

4.3 Conclusions

In the first part of the study, PKC isozyme-specific properties in the murine pulmonary vasculature were identified. Hypoxic pulmonary vasoconstriction was unaltered by loss of PKCα, but broad spectrum PKC inhibition markedly reduced HPV which has been proposed to involve activation of PKCε. Moreover, ET-1-induced vasoconstriction, an important feature of PAH pathophysiology, was promoted by PKCα, whereas PKCα deficiency or inhibition did not affect serotonin-evoked vasopressor response. Notably, PKCα deficiency led to vascular hyperresponsiveness towards the TXA2 analog U46619 and mRNA analysis revealed increased TP receptor expression in intrapulmonary arteries of *PKCα*$^{-/-}$ mice. Additionally, expression of atypical PKCι was upregulated. However PKCι inhibition had no effect on U46619-induced vasoconstriction concluding a minor functional role of PKCι upregualtion but major role of TP receptor upregulation for TXA2-induced hyperresponsiveness. Taken together, the PKC family with isozyme-selective properties might serve as a potential target in PAH therapy. However, coincidence of PKCα and TP receptor expression must be taken into consideration and requires further studies.

The finding of increased megaesophagus prevalence in young *PKCα*$^{-/-}$ mice, which is a characteristic of human achalasia, led to further investigations. The raised prevalence of esophageal dilation in juvenile *PKCα*$^{-/-}$ mice was associated with an increased portion of smooth muscle cells at the distal esophagus. However, inflammation, neuronal degeneration and fibrosis of the LES as typically observed in achalasia were not seen. Furthermore, analysis of PKC isozyme expression patterns in the LES of *PKCα*$^{-/-}$ mice revealed decreased expression of novel PKCδ and PKCε and atypical PKCζ and PKCι, which may influence LES basal tone. Since achalasia-like lesions were not present, this murine disorder does not adequately address the pathophysiology of human achalasia and therefore questions the value of an appropriate model for achalasia. Instead, it is assumed that megaesophagus in *PKCα*$^{-/-}$ mice appears as a consequence of delayed replacement of smooth muscle cells during esophageal maturation and therefore displays a functional muscular disorder rather than a neuronal achalasia-like issue.

In summary, the first hypothesis “PKCα modulates pulmonary vascular function” was supported since the mouse model showed differential involvement of PKCα in modulation of pulmonary vascular responsiveness. Beyond, the present data suggest that PKC is linked to TP receptor expression in murine intrapulmonary arteries. Hypothesis two “PKCα influences smooth muscle cell morphology of the LES, and loss of PKCα evokes changes in the LES which contribute to formation of megaesophagus” was confirmed partly. The random finding

of megeasophagus was associated with increased smooth muscle cells at the distal esophageal body with morphologically unchanged LES.

To conclude, results of this study provide evidence that the PKC family comprises highly specified members with isozyme-selective features. PKCα represents a multifunctional isozyme that plays a crucial role in smooth muscle cell function of pulmonary arteries, but also in mechanisms regulating esophageal smooth muscle cell distribution and probably esophageal maturation. Furthermore, the current data demonstrate that mutant mouse models are of considerable value for comprehensive research questions, but should be carefully and critically evaluated with respect to human conditions.

4.4 Outlook

Since changes in the PKC isozyme expression profile of the LES were present, motility studies should further define the impact of PKCα deficiency on the LES tonus. By esophageal manometry, LES function in mice displaying megaesophagus could be clarified. This technique is typically performed to evaluate disorders of motility and peristalsis by analyzing motor function of the esophageal body and sphincter and has been used in diverse achalasia studies in mice (Sivarao et al. 2001, Zizer et al. 2010, Muller et al. 2014).

Moreover, to investigate changes in smooth muscle cell distribution due to loss of PKCα, serial macroscopic and histological investigations during esophageal maturation, starting at embryonic stage, should be performed.

There is indispensable future work to do regarding the functional role of PKCα in the pulmonary vasculature. First of all the coincidence between PKCα and TP receptor expression should be verified. Is TP receptor upregulation a compensatory effect in the constitutive *PKCα*$^{-/-}$ mouse? What would that mean for long-term PKCα inhibition in PAH patients? To address these questions the effect of inducible PKCα deficiency on TP receptor expression and TXA2-induced vasoconstriction should be studied.

The present results provide evidence for a substantial and isozyme-specific role of PKC, in particular PKCα, in pulmonary vascular responsiveness. Therefore, future studies should focus on the effects of PKCα deficiency on pulmonary vascular resistance, hyperresponsiveness and pulmonary arterial remodeling - the hallmarks of PAH- by modeling the disease in a long-term experimental set-up.

5 Summary

Characterization of Protein Kinase C Alpha Deficiency in a Mouse Model

Elena Ariane Noe

The protein kinase C (PKC) familiy was first described in 1977 by Nishizuka and colleagues, the pioneers in PKC research, and more than 30 years ago its role in human diseases was recognized. From that time onward, researchers worldwide endeavored to unravel regulatory functions of PKC isozymes in numerous major disorders. PKC-mediated phosphorylation of serine/threonine residues controls the activation of multiple downstream proteins. This central role in signal transduction as well as ubiquitous expression of most of the 11 PKC members makes it challenging to define the complexity of isozyme specific PKC functions. Dependent on their structure and consequent way of activation, the PKC family is classified into conventional (PKCα, PKCβI, PKCβII, PKCγ), novel (PKCδ, PKCε, PKCθ, PKCη, PKCμ) and atypical (PKCζ, PKCι/PKCλ) PKC isozymes. The conventional PKCα is the most studied and best characterized isozyme, especially due to its crucial role in cancerogenesis. PKCα-evoked cellular proliferation, differentiation and apoptosis have been also proven and demonstrated in the pathogenesis of cardiovascular disorders. However, the exact role of PKCα in various pathologic conditions is still incompletely understood.

The human lung disease pulmonary arterial hypertension (PAH) is characterized by progressive changes in the morphology and function of pulmonary arteries. Several PKC isozymes including PKCα are known to modulate vascular smooth muscle function. Hence, the aim of the present work was to investigate PKC isozyme specific properties in the pulmonary vasculature following stimulation with vasoactive mediators that are important in the pathogenesis of PAH. For this purpose, effects of acute hypoxia, endothelin-1 (ET-1), serotonin and the thromboxane A2 (TXA2) analog U46619 were studies in isolated perfused and ventilated mouse lungs of PKCα deficient (*PKCα*$^{-/-}$) and corresponding wildtype (Dempsey et al.) mice. Broad spectrum PKC inhibition in WT mice was achieved with the non-selective PKC inhibitor bisindolymaleimide I (BIM), while usage of Gö6976 evoked selective PKC inhibition of conventional PKC isozymes PKCα and PKCβ.

In this study, acute hypoxic pulmonary vasoconstriction (HPV) was unaffected by loss of PKCα, whereas PKC inhibition with BIM significantly reduced HPV in WT mice. ET-1-induced vasopressor response, an important feature of PAH, was markedly attenuated by PKCα deficiency, non-selective and selective PKC inhibition suggesting a crucial role of PKCα in this scenario. In contrast, serotonin-evoked vasoconstriction was not affected by the absence of PKCα or by PKC inhibition with BIM. Notably, loss of PKCα caused pulmonary vascular hyperresponsiveness to the TXA2 analog U46619. mRNA expression analysis revealed

increased thromboxane A2 receptor levels in microdissected intrapulmonary arteries from naïve *PKCα*$^{-/-}$ mice. Besides, mRNA expression of atypical PKCι was upregulated in intrapulmonary arteries from *PKCα*$^{-/-}$ mice. However, since PKCι inhibition with sodium aurothiomalate hydrate (ATM) had no effect on TXA2-induced vasopressor response, the observed hyperresponsivness to TXA2 analog U46619 might not involve PKCι but certainly TP receptor upregulation.

Due to the random observation of esophageal dilation in *PKCα*$^{-/-}$ mice, further analyses were performed in 9-10-week-old and 14-month-old *PKCα*$^{-/-}$ and WT mice. Megaesophagus is a characteristic of human achalasia. The hallmark of achalasia is impaired smooth muscle cell relaxation of the lower esophageal sphincter (LES) due to neuronal degeneration of the esophageal myenteric plexus. Here, juvenile *PKCα*$^{-/-}$ mice showed a 60% prevalence of megaesophagus. With a prevalence of 10%, megaesophagi were present in aged *PKCα*$^{-/-}$ but also in WT mice independent of age. Histopathological investigations revealed an increased portion of the distal esophagus lined by smooth muscle cells in mice displaying meagaesophagus. However, achalasia-like inflammation, fibrosis or neuronal degeneration of the LES was not present. It was therefore hypothesized that loss of PKCα leads to delayed esophageal maturation due to failed replacement of embryonic smooth muscle cells by striated muscle cells. Since PKC isozyme expression profiles in LES of *PKCα*$^{-/-}$ mice showed decreased expression levels of four out of seven PKC isozymes, changes in the LES basal tone might have evoked smooth muscle cell hyperplasia at the distal esophagus leading to megaesophagus formation. Anyhow, PKCα deficiency appears to be associated with functional-muscular rather than neuronal achalasia-like pathological conditions.

In conclusion, the results gained from this work substantially increase the understanding of PKC isozyme selective properties in the pulmonary vasculature and provide new data on PKCα involvement in smooth muscle cell function of lung and esophagus. Future work should verify the functional role of PKCα in PAH pathogenesis by PAH modeling and LES function by manometric studies.

6 Zusammenfassung

Charakterisierung der Protein Kinase C Alpha Defizienz im Mausmodell

Elena Ariane Noe

Die Proteinkinase C (PKC)-Familie wurde erstmals 1977 von Nishizuka und Kollegen beschrieben und bereits vor mehr als 30 Jahren mit verschiedenen Krankheitsbildern assoziiert. Seitdem ist es Ziel von Wissenschaftlern weltweit die regulatorischen Funktionen der PKC-Isoenzyme in der Pathogenese von unterschiedlichen Erkrankungen zu erforschen und zu verstehen.

Die PKC-vermittelte Phosphorylierung von Serine/Threonine-Resten moduliert die Aktivierung zahlreicher nachgeschalteter Proteine. Diese zentrale Rolle in der Signaltransduktion sowie die ubiquitäre Expression der meisten 11 PKC Mitglieder machen Untersuchungen Isoenzym-spezifischer PKC-Funktionen zur komplexen Aufgabe. Anhand ihrer Struktur und Art der Aktivierung werden die Mitglieder der PKC-Familie in herkömmliche (PKCα, PKCβI, PKCβII, PKCγ), neuartige (PKCδ, PKCε, PKCθ, PKCη, PKCμ) und atypische Isoenzyme (PKCζ, PKCι/PKCλ) unterteilt. Die herkömmlichen PKCα ist das bisher am besten charakterisierte Isoenzym. PKCα-vermittelte zelluläre Proliferation, Differenzierung und Apoptose spielen eine Rolle in der Kanzerogenese, aber auch in der Pathogenese kardiovaskulärer Erkrankungen. Die exakte Rolle der PKCα in zahlreichen Erkrankungen ist jedoch noch nicht vollständig geklärt.

Die humane Lungenerkrankung pulmonalarterielle Hypertonie (PAH) zeichnet sich durch progressive morphologische und funktionelle Veränderungen der pulmonalen Arterien aus. Es ist bekannt, dass verschiedene PKC-Isoenzyme einschließlich der PKCα die Funktion vaskulärer glatter Muskelzellen regulieren. Ziel der vorliegenden Arbeit war daher die Untersuchung PKC-Isoenzym-spezifischer Funktionen im pulmonalen Gefäßsystem nach Einsatz vasokonstriktorischer Stimuli, die in der PAH-Pathogenese von Bedeutung sind. Dazu wurden die Effekte von akuter Hypoxie, Endothelin-1 (ET-1), Serotonin und dem Thromboxan A2 (TXA2) Analogon U46619 in isoliert perfundierte und ventilierte Lungen von PKCα-defizienten (*PKCα*$^{-/-}$) und korrespondierenden Wildtyp-Mäusen (Dempsey et al.) analysiert. Zur PKC Inhibition wurden der nicht-selektive Breitspektrum-PKC-Inhibitor Bisindolymaleimide I (BIM) und der für die konventionellen PKC Isoenzyme PKCα und PKCβ selektive Inhibitor Gö6976 in WT Mäusen eingesetzt.

Die hypoxisch pulmonalen Vasokonstriktion (HPV) blieb bei PKCα Defizienz unverändert, wohingegen eine pharmakologische PKC-Inhibition mittels BIM zu einer signifikanten verminderten HPV in WT-Mäusen führte. Untersuchungen der ET-1-induzierten vaskulären Reagibilität zeigten eine deutlich reduzierte ET-1 vermittelte Vasokonstriktion durch

PKCα-Defizienz ebenso wie durch nicht-selektive und selektive PKC-Inhibition. Im Gegensatz dazu blieb die Serotonin-induzierte Vasokonstriktion sowohl durch Fehlen von PKCα als auch durch PKC-Inhibition mittels BIM unverändert. Interessanterweise führte die Stimulation mit dem TXA2-Analogon U46619 zu einer pulmonalvaskulären Hyperreagibilität in PKCα-defizienten Mauslungen. Weiterführende mRNA-Analysen zeigten eine erhöhte Expression des Thromboxan A2-Rezeptors (Herve et al.) in mikrodissektierten intrapulmonalen Arterien naiver *PKCα*$^{-/-}$ Mäuse. Darüber hinaus wurde in *PKCα*$^{-/-}$ Mauslungen eine vermehrte mRNA Expression der atypischen PKCι gemessen. Eine selektive PKCι Inhibierung mittels Natriumaurothiomalat hatte jedoch keine Reduktion der U46619-vermittelten vaskulären Reagibilität zur Folge. Die beobachtete U46619-induzierte Hyperreagibilität in *PKCα*$^{-/-}$ Lungen scheint daher nicht PKCι vermittelt sondern primär bedingt durch die vermehrte TP Rezeptor Expression in intrapulmonalen Arterien zu sein.
Der zufällige Befund der Ösophagusdilatation in *PKCα*$^{-/-}$ Mäusen wurde in 9-10 Wochen alten und 14 Monate alten *PKCα*$^{-/-}$ und WT Mäusen genauer untersucht. Der Megaösophagus ist ein Charakteristikum der humanen Achalasie. Hauptmerkmal der Achalasie ist die fehlerhafte Relaxation des unteren Ösophagussphinkters (UÖS), verursacht durch neuronale Degenerationen des Plexus Myentericus. Juvenile *PKCα*$^{-/-}$ Mäuse zeigten eine 60%ige Megaösophagus-Prävalenz. Mit einer Prävalenz von 10% wurden Megaösophagi in 14 Monate alten *PKCα*$^{-/-}$, aber auch in WT-Mäusen altersunabhängig dokumentiert. Weiterführende histopathologische Untersuchungen der Megaösophagi zeigten einen vermehrten Anteil glatter Muskelzellen am distalen Ösophagusende. Achalasie-ähnliche Befunde wie Inflammation, Fibrose oder neuronale Degeneration des UÖS waren jedoch nicht vorhanden. Es ist anzunehmen, dass ein Verlust der PKCα zu einem fehlerhaften Umbau von embryonaler glatter zu quergestreifter Muskulatur und damit zu einer Verzögerung im Reifeprozess des Ösophagus führt. Da im UÖS *PKCα*$^{-/-}$ Mäuse eine verminderte Expression von vier von sieben PKC-Isoenzymen nachgewiesen wurde, könnte der Megaösophagus auch durch einen veränderten basalen Tonus des UÖS und einer daraus resultierenden Hyperplasie glatter Muskelzellen entstanden sein. Insgesamt deuten die Ergebnisse darauf hin, dass die PKCα-Defizienz eher mit funktionell-muskulären Störungen als Achalasie-ähnlichen Veränderungen assoziiert ist.

Die Ergebnisse dieser Studie liefern neue Erkenntnisse bezüglich der PKC-Isoenzym-selektiven Eigenschaften im pulmonalvaskulären Gefäßsystem sowie der Bedeutung der PKCα in glatten Muskelzellen in Lunge und Ösophagus. Zur Verifizierung der funktionalen Rolle der PKCα in der Pathogenese der PAH sowie zur genaueren Klärung der Bedeutung der PKCα für die UÖS Funktion sind weitere Studien notwendig.

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8 Appendix

Supplementary Table including Median, Minimum and Maximum Values of Δ Ppa mean

Pulmonary vascular responsiveness to thromboxane analog U46619, Endothelin-1 (ET-1), Serotonin and acute Hypoxia in WT and PKC$\alpha^{-/-}$ mice, with or without prior administration of broad spectrum PKC inhibitor bisindolylmaleimide I (BIM 1 × 10^{-6} mol/L), conventional PKC inhibitor Gö6976 (1 × 10^{-6} mol/L) and PKC iota inhibitor sodium aurothiomalate hydrate (ATM 2 × 10^{-5} mol/L). Data represent the difference in mean pulmonary arterial pressure (Δ Ppa mean shown in cmH2O*) as percentage of the maximum pressure response observed in the respective control group.

* Multiply with factor 0.0980665 in order to obtain values in kPa.

Stimulus:	**U46619** 3 × 10^{-8} mol/L		**U46619** 1 × 10^{-7} mol/L		**U46619** 3 × 10^{-7} mol/L	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of Values:	11	11	11	11	11	11
Minimum:	**15.61**	**11.84**	**38.22**	**27.99**	**62.98**	**43.6**
Median:	**22.07**	**17.49**	**43.87**	**45.22**	**72.94**	**70.52**
Maximum:	**43.06**	**37.68**	**115.7**	**82.36**	**193.2**	**132.4**
Strain, Treatment:	WT, solvent	WT, Gö 6976	WT, solvent	WT, Gö 6976	WT, solvent	WT, Gö 6976
Number of Values:	6	7	6	7	6	7
Minimum:	**12.07**	**12.07**	**42.25**	**46.48**	**94.16**	**89.34**
Median:	**15.09**	**14.49**	**51.31**	**59.76**	**98.39**	**119.5**

Maximum:	**26.56**	**19.92**	**71.23**	**71.23**	**109.9**	**139.4**
Strain:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$
Number of Values:	6	6	6	6	6	6
Minimum:	**9.94**	**22.2**	**32.47**	**71.23**	**63.28**	**142.8**
Median:	**17.06**	**34.29**	**53.34**	**97.74**	**98.23**	**172.1**
Maximum:	**26.17**	**59.97**	**80.51**	**146.1**	**128.9**	**274.3**
Strain Treatment:	PKC$\alpha^{-/-}$ solvent	PKC$\alpha^{-/-}$ ATM	PKC$\alpha^{-/-}$ solvent	PKC$\alpha^{-/-}$ ATM	PKC$\alpha^{-/-}$ solvent	PKC$\alpha^{-/-}$ ATM
Number of Values:	7	7	7	7	7	7
Minimum:	**6.14**	**8.187**	**31.38**	**42.98**	**60.04**	**74.37**
Median:	**8.187**	**13.65**	**40.25**	**57.31**	**92.11**	**124.2**
Maximum:	**16.37**	**24.56**	**83.24**	**105.8**	**161.7**	**254.5**

Stimulus:	**ET-1** 1 × 10^{-8} mol/L		**ET-1** 1 × 10^{-7} mol/L	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of Values:	6	5	6	5
Minimum:	**5.13**	**3.18**	**80.86**	**53.01**
Median:	**9.04**	**6.12**	**88.8**	**61.81**
Maximum:	**16.12**	**10.5**	**132.2**	**81.6**

Strain, Treatment:	WT, solvent	WT, Gö 6976	WT, solvent	WT, Gö 6976
Number of Values:	4	4	4	4
Minimum:	**5.65**	**5.2**	**89.44**	**71.6**
Median:	**7.0**	**7.45**	**96.78**	**74.2**
Maximum:	**9.03**	**9.49**	**117**	**89.44**

Strain:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$
Number of Values:	7	6	7	6
Minimum:	**2.64**	**1.39**	**52.9**	**14.58**
Median:	**4.17**	**3.75**	**100.8**	**55.19**
Maximum:	**26.93**	**8.05**	**141.5**	**122.9**

Stimulus:	**Serotonin** 3 × 10^{-6} mol/L		**Serotonin** 1 × 10^{-5} mol/L		**Serotonin** 3 × 10^{-5} mol/L	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of Values:	8	7	8	7	8	7
Minimum:	**21.43**	**17.01**	**42.18**	**29.59**	**52.04**	**36.73**
Median:	**44.22**	**43.54**	**70.75**	**74.83**	**93.37**	**90.48**
Maximum:	**65.31**	**89.12**	**138.4**	**165.6**	**198.3**	**185.7**

Strain:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$
Number of Values:	7	7	7	7	7	7
Minimum:	**12.24**	**21.77**	**33.33**	**38.78**	**55.78**	**55.1**
Median:	**25.17**	**31.97**	**57.14**	**62.59**	**85.03**	**91.84**
Maximum:	**138.1**	**72.79**	**151.7**	**162.6**	**198**	**216.3**

Stimulus:	**Hypoxia 1%**			
Strain, Treatment:	WT, solvent	WT, BIM	WT	PKCa$^{-/-}$
Number of Values:	5	5	8	9
Minimum:	**78.81**	**44.66**	**70.73**	**57.32**
Median:	**91.07**	**63.05**	**100**	**104.9**
Maximum:	**127**	**73.56**	**125.6**	**131.7**

Stimulus:	**Normoxia**			
Perfusion Rate:	0.5 mL × min -1		1 mL × min -1	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of values:	5	5	5	5
Minimum:	**-35.57**	**-34.26**	**-1.32**	**-2.64**
Median:	**-27.01**	**-27.01**	**0**	**-0.66**
Maximum:	**-26.35**	**-25.69**	**0**	**0**
Perfusion Rate:	1.5 mL × min -1		2 mL × min -1	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of values:	5	5	5	5
Minimum:	**17.13**	**15.81**	**28.99**	**27.67**

Median:	**18.45**	**18.45**	**30.3**	**31.62**
Maximum:	**20.42**	**23.06**	**36.23**	**41.5**

Stimulus:	**Hypoxia 1%**			
Perfusion Rate:	0.5 mL × min -1		1 mL × min -1	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of Values:	5	5	5	5
Minimum:	**3.294**	**-19.76**	**40.84**	**16.47**
Median:	**21.74**	**-14.49**	**67.19**	**19.76**
Maximum:	**29.64**	**-9.881**	**79.05**	**32.28**
Perfusion Rate:	1.5 mL × min -1		2 mL × min -1	
Strain, Treatment:	WT, solvent	WT, BIM	WT, solvent	WT, BIM
Number of Values:	5	5	5	5
Minimum:	**54.02**	**32.28**	**61.26**	**46.77**
Median:	**91.57**	**40.84**	**103.4**	**51.38**
Maximum:	**106.1**	**46.11**	**119.9**	**63.24**

Stimulus:	**Normoxia**			
Perfusion Rate:	0.5 mL × min -1		1 mL × min -1	
Strain, Treatment:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$

Number of Values:	8	9	8	9
Minimum:	**-40.57**	**-39.76**	**-6.49**	**-4.06**
Median:	**-34.08**	**-32.45**	**0.81**	**0**
Maximum:	**-22.72**	**-21.1**	**4.89**	**6.49**

Perfusion Rate:	1.5 mL × min -1		2 mL × min -1	
Strain. Treatment:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$
Number of Values:	8	9	8	9
Minimum:	**16.23**	**14.6**	**34.08**	**29.21**
Median:	**25.56**	**25.96**	**41.38**	**37.32**
Maximum:	**27.59**	**36.51**	**47.87**	**49.49**

Stimulus:	**Hypoxia 1%**			
Perfusion Rate:	0.5 mL × min -1		1 mL × min -1	
Strain. Treatment:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$
Number of Values:	8	9	8	9
Minimum:	**-17.04**	**-16.23**	**25.15**	**16.23**
Median:	**5.68**	**6.49**	**49.09**	**47.87**
Maximum:	**57.61**	**58.42**	**96.55**	**73.02**

Perfusion Rate:	1.5 mL × min -1		2 mL × min -1	
Strain. Treatment:	WT	PKC$\alpha^{-/-}$	WT	PKC$\alpha^{-/-}$
Number of Values:	8	9	8	9
Minimum:	**48.68**	**30.83**	**63.29**	**44.62**
Median:	**78.7**	**88.44**	**94.93**	**97.36**
Maximum:	**120.9**	**118.5**	**144.4**	**153.3**

9 Pre-Publications:

Oral Poster Presentations:

C. Tabeling, **E. Noe,** J. Naujoks, J. M. Doehn, S. Hippenstiel, B. Opitz, N. Suttorp, R. Klopfleisch, M. Witzenrath.

PKCalpha deficiency in mice is associated with pulmonary vascular hyperresponsiveness to thromboxane A2 via increased TXA2 receptor expression. Eur Respir J 2014; 44: Suppl. 58, 308

European Respiratory Society (ERS) International Congress, September 7th 2014 Munich, Germany

C. Tabeling, **E. Noe,** J. Naujoks, J. M. Doehn, S. Hippenstiel, B. Opitz, N. Suttorp, R. Klopfleisch, M. Witzenrath.

PKCalpha deficiency in mice is associated with pulmonary vascular hyperresponsiveness to thromboxane A2 via increased TXA2 receptor expression.

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 15.02.2016 Elena Noe