Title page information

Role of $G_{i/o}$ -Src kinase-PI3K/Akt pathway and caveolin-1 in β_2 -adrenoceptor coupling to endothelial NO Synthase in mouse pulmonary artery

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Abstract

Activation of the β_2 -adrenoceptor (β_2 -AR) elicits an endothelial nitric oxide synthase (eNOS)-dependent relaxation in mouse pulmonary artery, which, contrary to the muscarinic receptor-dependent relaxation, is preserved in hypoxic pulmonary arterial hypertension. We therefore characterized the signaling pathways underlying the β_2 -AR-mediated eNOS activation, with special focus on $G_{i/o}$ proteins, protein kinases and caveolae. Functional studies (for evaluation of vasorelaxant responses), Western blots (for assessment of eNOS and caveolin-1 phosphorylations) and transmission electron microscopy (for visualization of caveolae) were conducted in pulmonary arteries from wild-type or caveolin-1 knockout mice. In wild-type isolated arteries, relaxation to the selective β_2 -AR agonist procaterol was reduced by inhibitors of $G_{i/o}$ proteins (pertussis toxin, PTX), phosphatidylinositol 3-kinase (PI3K; wortmannin or LY 294002), Akt (Akt inhibitor X) and Src-kinase (PP2) and by cholesterol depletion (using methyl- β -cyclodextrin). **Procaterol induced eNOS phosphorylation at Ser¹¹⁷⁷, which was prevented by PTX, PP2 or Akt inhibitor, as well as caveolin-1 phosphorylation at Tyr¹⁴, which was decreased by PTX or PP2.** Caveolin-1 gene deletion resulted in endothelial caveolae disruption in mouse pulmonary artery and in potentiation of procaterol-induced relaxation. Unlike procaterol, acetylcholine-induced relaxation was unaffected by PTX, methyl- β -cyclodextrin or caveolin-1 gene deletion. To conclude, **the mouse pulmonary endothelial β_2 -AR is coupled to a $G_{i/o}$ -Src kinase-PI3K/Akt pathway to promote eNOS phosphorylation at Ser¹¹⁷⁷ leading to a NO-dependent vasorelaxation, and caveolin-1 exerts a negative control on this response, which is removed by its phosphorylation at Tyr¹⁴ through a $G_{i/o}$ -Src kinase pathway.** Since pulmonary β_2 -AR- and muscarinic receptor-mediated relaxations differentiate in their respective signaling pathways leading to eNOS activation and susceptibilities during hypoxia-induced pulmonary arterial hypertension, mechanisms underlying eNOS activation might be key determinants of pulmonary endothelial dysfunction.

Keywords: Pulmonary artery; Endothelium; β_2 -adrenoceptor; Nitric Oxide Synthase; Caveolin.

Abbreviations: AMPK, AMP-activated protein kinase; Cav-1 KO, caveolin-1 knockout mice; eNOS, endothelial NO synthase; HUVEC, human umbilical vein endothelial cells; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, Dimethyl Sulfoxide; **JNK, c-Jun N-terminal kinase**; L-NAME, *N*^ω-nitro-L-arginine methylester; MAPK, mitogen-activated protein kinases; NO, nitric oxide; PGF_{2α}, prostaglandin F_{2α}; PKA, cAMP-dependent protein kinase; PI3K, phosphatidylinositol 3-kinase; PTX, pertussis toxin; WT, wild-type.

1. Introduction

Nitric oxide (NO), produced by the endothelial NO synthase (eNOS), is a major vasculoprotective agent owing to its vasodilator, anti-aggregant, anti-oxidant and anti-proliferative properties [1]. Endothelial dysfunction, and particularly an impaired endothelial NO production and/or availability, plays a key role in many cardiovascular risk factors and diseases, including pulmonary arterial hypertension [2]. Pulmonary arterial hypertension is a severe disease, leading to right ventricular hypertrophy followed by right ventricular failure and ultimately death. The increase in pulmonary arterial resistances is the consequence of both structural remodelling and alterations of vasomotor responses, related to the imbalance between vasodilator/antiproliferative and vasoconstrictor/proliferative factors [3]. Restoration of NO production and/or availability in pulmonary arteries appears to be an interesting therapeutic approach to treat this disease.

As in other cardiovascular diseases, endothelial dysfunction in models of pulmonary arterial hypertension is established when pulmonary arteries exhibit a decrease in the relaxant response to acetylcholine, the reference endothelium-dependent vasodilator, acting through the activation of endothelial muscarinic receptors coupled to NO production [4,5]. In a mouse model of chronic hypoxia-induced pulmonary arterial hypertension, we have shown that the relaxant response induced by the β_2 -AR stimulation, which is also endothelium- and eNOS-dependent, is preserved, whereas the relaxant response to acetylcholine is decreased [5,6]. The preservation of this pulmonary endothelium-dependent β_2 -adrenergic vasorelaxation highlights the potential interest of the β_2 -AR as a therapeutic target in pulmonary arterial hypertension and suggests that the mechanisms underlying eNOS activation are determinant for expression of endothelial dysfunction in pulmonary arteries. Although β_2 -ARs have been

described as endothelial β -ARs associated with a NO-dependent relaxant response in some systemic vascular beds [7-9], there is limited information about the molecular mechanisms underlying the β_2 -adrenergic-mediated activation of eNOS.

The regulation of eNOS activity involves complex molecular mechanisms, including not only the calcium/calmodulin-dependent pathway, but also posttranslational mechanisms especially through modifications of eNOS phosphorylation and eNOS-protein interactions [10,11]. In particular, caveolin-1, a major protein of endothelial caveolae, interacts with the inactive state of eNOS and the dissociation of this complex increases eNOS activity [12,13]. Whereas in mouse pulmonary arteries muscarinic-dependent activation of eNOS involves the M_3 subtype [14], which is coupled to a G_q /phospholipase C pathway and subsequent increase in cytosolic calcium concentration in endothelial cells, it is worth noting that the coupling mechanism between β_2 -AR and eNOS remains unknown in these arteries, except that it is independent of a cAMP/cAMP-dependent protein kinase (PKA) pathway [6], suggesting that G_s proteins are not recruited.

The aim of this study was therefore to identify the components of the signaling pathway involved in eNOS activation elicited by endothelial β_2 -AR stimulation and responsible for the β_2 -AR-mediated relaxation in the mouse pulmonary artery. Specifically, the following issues were addressed: (i) to evaluate the role of $G_{i/o}$ proteins in the β_2 -adrenergic relaxant response, as β_2 -ARs are known to couple not only to G_s but also to $G_{i/o}$ proteins, in several tissues including cardiovascular ones [15,16]; (ii) to determine the effect of β_2 -AR stimulation on eNOS phosphorylation, at the key activating Ser¹¹⁷⁷ residue of eNOS [17,18], and identify the protein kinases involved in eNOS phosphorylation; (iii) to investigate the role of caveolae and caveolin-1 in the mouse pulmonary β_2 -adrenergic relaxant response.

The contribution of these different signaling molecules to the pulmonary endothelial β_2 -AR-mediated activation of eNOS was investigated by using pharmacological tools and a genetic approach with caveolin-1 knockout mice (Cav-1 KO). In some experiments, the β_2 -adrenergic response was compared to the reference muscarinic relaxation which is also NO-dependent [5].

2. Material and Methods

The investigation is in accordance with the EC Directive 86/609/EEC on the "Protection of animals used for experimental and other scientific purposes". Agreement (number A 33409) was obtained from French authorities.

2.1. Reagents and antibodies

In the absence of indication, drugs were obtained from Sigma-Aldrich (St. Quentin, Fallavier, France). Procaterol was from Tocris Bioscience (Bristol, UK). Pertussis toxin (PTX) and all kinases inhibitors (Akt inhibitor X, compound C, LY 294002, PD 98059, PP2, SB 203580, **SP 600125**, U0126, wortmannin) were from Calbiochem (Merck Chemicals Ltd, Nottingham, UK). Prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) (dinoprost tromethamine, Dinoprost[®]) was obtained from Pfizer (Paris, France), the phosphatase inhibitor cocktail from Thermo Fisher Scientific (Cergy Pontoise, France), Dulbecco's Modified Eagle's Medium (DMEM) from Gibco (Invitrogen, Cergy Pontoise, France) and NuPAGE[®] from Invitrogen.

Pharmacological agents (PTX, agonists receptors, kinases inhibitors and methyl- β -cyclodextrin) were prepared in distilled water except compound C, LY 294002, PD 98059, PP2, SB 203580, **SP 600125**, U0126 and wortmannin which were prepared in Dimethyl Sulfoxide (DMSO). Drugs concentrations were chosen according to their pharmacological properties (affinity and selectivity) described in the literature.

Primary antibodies were anti-eNOS (monoclonal, BD Transduction Laboratories, San Jose, CA, USA), anti-phospho-eNOS (Ser¹¹⁷⁷) (polyclonal; Cell Signaling Technology, Beverly, MA, USA), anti-caveolin-1 (polyclonal; BD Transduction Laboratories), anti-phospho-caveolin-1 (Tyr¹⁴) (polyclonal; Cell Signaling Technology), anti- β -actin (monoclonal; Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies were obtained from

Jackson Immunoresearch Laboratories (Suffolk, UK; for eNOS and phospho-eNOS detection) or Thermo Fisher Scientific (for caveolin-1 and β -actin detection).

2.2. Animals and tissues

Male C57BL6 mice (8-12 weeks old) were purchased from Elevage Janvier (Le Genest St Isle, France). Some experiments were conducted in male Cav-1 KO mice and their wild-type littermates (WT, C57BL6-background) (5-6 months old; a gift from Dr. C. Dessy, Unit of Pharmacology and Therapeutics, Université Catholique de Louvain, Brussels, Belgium).

Left and right branches of the extralobar pulmonary artery were removed and cleaned of adventitial tissue [5]. These vessels were immediately used for functional and biochemical studies, except those used for the evaluation of the role of $G_{i/o}$ proteins, which were incubated in DMEM in the absence or presence of 400 ng/ml PTX for 12 hours at 37°C in a humidified atmosphere (95% air / 5% carbon dioxide).

2.3. Vascular reactivity studies

Segments of pulmonary arteries were mounted in a wire myograph (Multi Myograph System, model 610M, J.P. Trading, Aarhus, Denmark) to measure isometric tension, as previously described [5,6]. Relaxant effects of 10 μ M acetylcholine and 0.1 μ M procaterol (in the presence of the α -adrenoceptor antagonist phentolamine, 10 μ M) were studied in the same artery, after submaximal pre-contraction with $\text{PGF}_{2\alpha}$. To characterize the signaling pathway involved in vasorelaxation, arteries were pretreated before addition of $\text{PGF}_{2\alpha}$ in the absence or presence of the cholesterol-depleting agent (1 mM methyl- β -cyclodextrin) or enzyme inhibitors [for phosphatidylinositol 3-kinase (PI3K; 10 μ M LY 294002 or 0.1 μ M wortmannin), Akt (5 μ M Akt inhibitor X), Src-kinase (10 μ M PP2), mitogen-activated protein kinases (MAPK) pathway (20 μ M PD 98059, 10 μ M UO126, 5 μ M SB 203580 or 10 μ M SP

600125), AMP-activated protein kinase (AMPK; 2 μ M compound C), or NOS (300 μ M *N*^ω-nitro-L-arginine methylester, L-NAME)]. In experiments performed in the presence of pharmacological agents diluted in DMSO, control arteries were incubated in the presence of the equivalent concentration of DMSO.

Relaxant effects induced by procaterol and acetylcholine are expressed as percentage of relaxation of the tone induced by PGF_{2α}. The area of the relaxant effect, measured over 15 min, was evaluated with Origin 6 software (Microcal Inc, Piscataway, NJ, USA) and reported to the amplitude of contraction induced by PGF_{2α}.

2.4. Western blot

To evaluate the effect of β_2 -AR stimulation on phosphorylation of eNOS and caveolin-1, pulmonary arteries were placed in the myograph chambers. After pretreatment in the absence or presence of kinase inhibitors (as described in vascular reactivity studies), arteries were incubated with 10 μ M PGF_{2α} and 10 μ M phentolamine for 30 min, before addition of solvent or procaterol (0.1 μ M) for 30 sec or 5 min. The arteries were then quickly frozen in liquid nitrogen, and stored at -80°C. To evaluate the expression of caveolin-1, pulmonary arteries isolated from Cav-1 KO and WT mice were frozen immediately after their removal from animals.

Arteries were homogenized in cold lysis buffer [containing: 1% sodium dodecyl sulfate (SDS), 10⁻² M Tris-HCl (pH 7.4), 5 μ g/ml aprotinin, 2.5 μ g/ml leupeptin and phosphatase inhibitor cocktail] for 45 min on ice followed by brief sonication. Tissue lysates were centrifuged (15 000 g for 10 min at 4°C) and the supernatant was resuspended in the loading buffer (NuPAGE[®] with 5% β -mercaptoethanol). Proteins were resolved by electrophoresis (Mini Gel Protean III System, Bio-Rad Laboratories, Hercules, CA, USA) in a 10% Tris-Glycine gel and transferred on Hybond ECL membranes (Amersham Biosciences,

Piscataway, NJ, USA) for 45 minutes at 100 V (Minitrans-blot Cell, Bio-Rad Laboratories). Membranes were blocked overnight at 4°C. After incubation with the appropriate primary antibody, membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using a chemiluminescence detection kit (Lumi-Light, Roche Diagnostics, Meylan, France).

2.5. Transmission Electronic Microscope Examination

Pulmonary arteries isolated from Cav-1 KO and WT mice were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hours at 4°C, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hour at room temperature, dehydrated through a series of graded ethanol and propylene oxide, and embedded in a mixture of propylene oxide and Epoxy resin (v/v) for 2 hours and then in 100% Epon resin. The resin was polymerized at 60°C for 48 hours. The samples were sectioned using a diamond knife on an ultra-microtome (Ultracut-E, Leica Microsystems). Thin sections (70 nm) were picked up on copper grids and then stained with uranyl acetate and lead citrate. The grids were examined with a transmission electron microscope at 120kV (Tecnai 12, FEI).

2.6. Statistical analysis

Data are given as mean $\pm$ S.E.M. of n experiments (n : number of mice). Different parameters were compared using Student's t -test for paired or unpaired data, where appropriate. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Role of $G_{i/o}$ proteins in the β_2 -AR-mediated relaxant response in mouse pulmonary artery

In a previous study, we have shown that in mouse pulmonary artery precontracted by PGF_{2 α} , 0.1 μ M procaterol (in the presence of phentolamine) induces a relaxation which is exclusively due to the endothelial β_2 -AR, eNOS and soluble guanylyl-cyclase stimulation [6]. As shown in Figure 1, a pretreatment of arteries with PTX, a $G_{i/o}$ protein inhibitor, significantly reduced the relaxation to procaterol without affecting the relaxation elicited by acetylcholine, the muscarinic agonist. This suggests that the β_2 -AR-mediated relaxant response involves activity of $G_{i/o}$ proteins.

3.2. Role of eNOS phosphorylation in the β_2 -AR-mediated relaxant response in mouse pulmonary artery

The phosphorylation status of eNOS is a critical determinant of its activity. As numerous kinases (including PI3K/Akt, PKA, AMPK, MAPK cascade, Src-kinase) may either directly or indirectly contribute to eNOS activation depending on the stimulus [10,11], we evaluated the effect of pharmacological inhibitors of these different pathways on the pulmonary β_2 -AR-mediated relaxant response. Two PI3K inhibitors (wortmannin or LY 294002), the Akt inhibitor X and the Src-kinase inhibitor PP2 significantly decreased the amplitude of relaxation induced by procaterol in mice pulmonary arteries (Fig. 2A, B, D). The concomitant application of LY 294002 and PP2 further decreased the response to procaterol (76% inhibition with LY 294002 + PP2 compared 41% and 46% inhibition with LY 294002 and PP2, respectively; $P<0.05$). By contrast, the inhibitors of ERK1/2 pathway (UO126 or PD 98059), p38-MAPK (SB 203580), c-Jun N-terminal kinase (JNK; SP 600125) and AMPK

(compound C) have no effect on this procaterol-mediated relaxant response (Fig. 2A, C, E). This suggests that in mouse pulmonary arteries, PI3K/Akt and Src-kinase, but not ERK1/2, p38-MAPK, JNK or AMPK, pathways participate in the β_2 -adrenergic relaxation.

The PI3K/Akt pathway is known to directly phosphorylate the key Ser¹¹⁷⁷ residue of eNOS, leading to its activation [17,18], whereas Src-kinase has been reported to act upstream of PI3K/Akt [19,20]. We then determined the effect of 0.1 μ M procaterol (in the presence of PGF_{2 α} to mimic the experimental conditions of vascular reactivity studies) on eNOS phosphorylation at Ser¹¹⁷⁷ in mouse pulmonary arteries. As shown in Figure 3A, stimulation of mouse pulmonary arteries with procaterol increased the phosphorylation of eNOS at Ser¹¹⁷⁷. This effect was observed after a 30 sec-incubation and further increased after 5 min to become statistically significant. Both Akt inhibitor X and PP2 abolished the effect of procaterol on the phosphorylation of eNOS at Ser¹¹⁷⁷ (Fig. 3B). In PTX-pretreated arteries, procaterol failed to modify eNOS phosphorylation at Ser¹¹⁷⁷ (Fig. 3B).

3.3. Role of caveolae and caveolin-1 in the β_2 -AR-mediated relaxant response in mouse pulmonary artery

To examine the role of caveolae in the β_2 -adrenergic-dependent relaxation, pulmonary arteries were incubated in the presence of methyl- β -cyclodextrin, a cholesterol-depleting agent that disrupts caveolae [21,22]. As shown in Figure 4, methyl- β -cyclodextrin treatment resulted in a 50% reduction of the procaterol-induced relaxation but had no significant effect on the endothelium-dependent relaxation to acetylcholine.

The role of caveolin-1, an essential structural protein of endothelial caveolae, was further addressed in pulmonary arteries isolated from Cav-1 KO mice. The loss of caveolin-1 protein expression in Cav-1 KO pulmonary arteries (Fig. 5A) was associated with a disruption of caveolae at the endothelial cell membranes (Fig. 5B). As illustrated in Figure 5C, the

relaxation to procaterol was amplified and sustained in duration in Cav-1 KO compared to WT pulmonary arteries. On average, Cav-1 gene disruption increased the relaxant effect to procaterol by 179% in area (Fig. 5D) and 39% in amplitude (Fig. 5E) compared to WT pulmonary arteries. By contrast, the relaxant response to acetylcholine was similar in terms of amplitude and duration in WT and Cav-1 KO pulmonary arteries (Fig. 5D & E). As in WT mice pulmonary arteries, both procaterol and acetylcholine-induced relaxations were markedly decreased by L-NAME, a NOS inhibitor, in Cav-1 KO mice pulmonary arteries (Fig. 5E). Furthermore, we have observed that PTX treatment significantly decreased the relaxation to procaterol in Cav-1 KO mice pulmonary arteries [$63.8 \pm 4.6\%$ relaxation in control arteries (n=6) compared to $39.5 \pm 8.2\%$ in PTX-treated arteries (n=6); $P < 0.05$; *data not shown*]. Altogether, these results suggest that the β_2 -AR coupling to Gi/o protein is preserved in the absence of Cav-1 and that caveolin-1 exerts a negative control on the β_2 -adrenergic relaxant pathway.

Src-kinase is known to phosphorylate caveolin-1 at Tyr¹⁴ [23]. We therefore investigated the effect of β_2 -AR stimulation on caveolin-1 phosphorylation at this residue. Incubation of pulmonary arteries with procaterol caused a transient increase in caveolin-1 phosphorylation at Tyr¹⁴ which was clearly seen at 30 sec- (Fig. 6) but disappeared at 5 min-incubation (*data not shown*). The level of caveolin-1 phosphorylation induced by a 30 sec-stimulation with procaterol was decreased by more than 50% not only in the presence of PP2, the Src-kinase inhibitor, but also in PTX-pretreated arteries (Fig. 6).

4. Discussion

Endothelial β_2 -AR stimulation elicits a NO-dependent relaxant response in both systemic [7-9] and pulmonary [6] vasculature. However, the molecular mechanisms involved in the coupling of β_2 -ARs to eNOS activation remained poorly understood. The present study depicts accurately the signaling cascade of the mouse pulmonary β_2 -AR. We report that the endothelial β_2 -adrenergic-mediated pulmonary relaxation involves a $G_{i/o}$ protein/Src-kinase/PI3K/Akt pathway responsible for eNOS activation through its phosphorylation at the Ser¹¹⁷⁷ residue. Interestingly, our results provide evidence for a tight control of this pathway by caveolin-1.

Historically, β_2 -ARs were classified as G_s -protein coupled receptors. However, it is now widely recognized that β_2 -ARs can also efficiently couple to $G_{i/o}$ proteins. Our previous study demonstrated that the relaxation induced by β_2 -AR stimulation in mouse pulmonary artery is independent of the cAMP/PKA pathway [6], which strongly argues against the involvement of G_s proteins. In this study, we found that the mouse pulmonary β_2 -adrenergic vasorelaxant response was decreased by PTX, which is known to ADP-ribosylate $G_{i/o}$ proteins in arterial preparations [24]. The fact that PTX failed to alter the relaxant response to the muscarinic agonist acetylcholine, which likely involves activation of G_q proteins, confirmed the specificity of PTX in our experimental conditions. Our results indicate that mouse pulmonary endothelial β_2 -AR couples to $G_{i/o}$ proteins to trigger the signaling cascade involved in NO-dependent relaxation. PTX treatment decreased the β_2 -adrenergic response by only 50%. This might be explained either by the partial inhibition of $G_{i/o}$ proteins in our experimental conditions, or by the contribution of other G proteins in the β_2 -adrenergic vasorelaxant response. However, such a coupling of β_2 -ARs to $G_{i/o}$ proteins might be the standard coupling

in endothelial cells as it has also been recently reported in human umbilical vein endothelial cells (HUVEC) [25] and in rat carotid artery [16].

Phosphorylation of eNOS at the Ser¹¹⁷⁷ residue is now recognized as a major mechanism of eNOS activation following diverse stimuli such as shear stress or receptor agonists [26]. However, the effect of endothelial β_2 -AR stimulation on eNOS phosphorylation state has been little studied. In rat aorta and cultured HUVEC, β_2 -AR stimulation has been reported to increase the phosphorylation of eNOS at the Ser¹¹⁷⁷ in a PKA- and PI3K/Akt-dependent manner [9,25]. In this study, we found that in mouse pulmonary artery, β_2 -AR activation promoted the phosphorylation of eNOS at the Ser¹¹⁷⁷, with kinetics consistent with the resulting vasorelaxation. We also demonstrated that **PTX**, Src kinase inhibitor and Akt inhibitor **not only abolished** the β_2 -adrenergic-mediated eNOS phosphorylation on Ser¹¹⁷⁷, **but also reduced the** vasorelaxant response related to the **β_2 -AR stimulation**. By contrast, inhibitors of ERK1/2, p38-MAPK, **JNK** and AMPK (present study), and also of the cAMP/PKA pathway [6], did not modify the β_2 -adrenergic response. Taken together, these results indicate that in mouse pulmonary artery, the β_2 -adrenergic relaxant response **partly** involves eNOS phosphorylation at Ser¹¹⁷⁷ through **a G_{i/o} proteins-**, Src kinase- and PI3K/Akt-dependent, but cAMP/PKA-, MAPK- and AMPK-independent pathway. Such a β_2 -AR coupling to the PI3K/Akt pathway [9,27,28] or to the Src kinase pathway [16,29] has been previously reported to participate in the β_2 -AR signaling in different cell types, including endothelial cells. However, in this study we show for the first time that both pathways contribute together to the β_2 -AR triggered eNOS phosphorylation and subsequent vasorelaxation. Our data suggest that, upon β_2 -AR activation, Akt is the kinase which directly phosphorylates eNOS at Ser¹¹⁷⁷, as widely recognized in different cell types [26], whereas the

Src tyrosine kinase likely acts upstream of PI3K/Akt, as clearly described in the endothelial estrogen receptor signaling cascade [19,30].

The fact that PTX, Src kinase inhibitor and Akt inhibitor abolished the β_2 -adrenergic effect on eNOS phosphorylation at Ser¹¹⁷⁷, but only partly reduced its vasorelaxant response, whereas this β_2 -adrenergic relaxant response has been shown to be totally eNOS-dependent [6], suggests that other mechanism(s) than eNOS phosphorylation at Ser¹¹⁷⁷ are involved in eNOS activation triggered by β_2 -AR stimulation in mouse pulmonary artery. Interestingly, we have shown that Src kinase and Akt inhibitors exert additive inhibitory effects on the β_2 -adrenergic relaxant response. Furthermore, Src kinase is known to exert a striking role in eNOS-dependent signaling related to its ability to directly phosphorylate caveolin-1 at Tyr¹⁴ [23] and Src kinase-dependent phosphorylation of caveolin-1 has recently been proposed to promote caveolin-1 dissociation from eNOS, leading to eNOS activation and caveolae-mediated endocytosis [31,32]. In this context, we focused on the potential role of caveolin-1 in the additional mechanism responsible for β_2 -AR-dependent eNOS activation. Here, we reported for the first time that β_2 -AR stimulation induced a rapid and transient increase in caveolin-1 phosphorylation at Tyr¹⁴, which was reduced by PTX or Src kinase inhibitor. Thus, this suggests that in mouse pulmonary artery, the G_{i/o} proteins/Src kinase pathway contributes to eNOS activation not only through its phosphorylation at Ser¹¹⁷⁷, but also through a caveolae-dependent mechanism. As the inhibitory effect of PTX and PP2 was only partial on the β_2 -AR-mediated caveolin-1 phosphorylation but total on the β_2 -AR-mediated eNOS phosphorylation, we can not exclude that another signaling pathway contributes to the increased caveolin-1 phosphorylation. This would deserve further investigation.

We therefore provided additional evidence for a role of caveolin-1 in the mouse pulmonary β_2 -AR-elicited relaxant response by using two different models of caveolae disruption.

Pharmacological disruption of caveolae by methyl- β -cyclodextrin, a cholesterol-binding agent [21], decreased the β_2 -AR-mediated relaxant response, whereas the genetic disruption of caveolae, by using arteries isolated from Cav-1 KO mice, increased the β_2 -AR response, which was still **Gi/o proteins-dependent, as shown by its inhibition in arteries treated with PTX, and** eNOS-dependent, as shown by its abolition in the presence of the NOS inhibitor L-NAME. The apparent discrepancy in the vascular functional consequence of pharmacological or genetic caveolae disruption has been recently discussed [33]. According to these authors, caveolin-1 protein expression persists after methyl- β -cyclodextrin treatment, and acts as a scavenger of eNOS protein, inhibiting its activity, whereas in Cav-1 KO arteries, eNOS protein becomes free and more active. Our results are consistent with - and further support - this model, as it is the first time, to our knowledge, that the effect of both pharmacological and genetic disruptions was investigated in parallel on a NO-dependent vasorelaxation in a given artery. This point is important because vasorelaxant responses studied in arteries isolated from Cav-1 KO mice have been shown to be decreased, increased or not modified, depending on the vasorelaxant agent or the vascular bed studied [32,34-36]. Regarding the β_2 -AR-mediated vasorelaxation, Neidhold *et al.* reported that it was not modified in the saphenous artery from Cav-1 KO mouse [37]. However, the contribution of the endothelium to this relaxant response was not investigated and the authors assumed it to be smooth muscle-dependent. Thus, a major finding of our study is that endothelial caveolin-1 plays a key role in controlling the β_2 -AR-dependent activation of eNOS in mouse pulmonary artery, **but is not required for β_2 -AR coupling to Gi/o proteins.** Even if we can not conclude about the localization of β_2 -ARs in the pulmonary endothelial caveolae, the latter appear to be non-essential structures in the β_2 -AR signaling pathway, as suggested by data obtained in Cav-1 KO mice.

Contrary to the β_2 -AR-mediated response, the muscarinic receptor-mediated relaxation of mouse pulmonary arteries, which is also fully NO-dependent, was not affected by either pharmacological or genetic disruptions of endothelial caveolae. Acetylcholine-induced relaxation has been shown to be decreased after membrane cholesterol depletion in different rat arteries [38,39], but either increased or not modified in systemic arteries isolated from Cav-1 KO mice [34,36,37]. These differences are currently unexplained. Our results highlight the fact that the role of caveolae in NO-dependent relaxant responses may vary from one vascular bed to another. They also suggest that in mouse pulmonary artery, the endothelial muscarinic receptor regulates a pool of eNOS which is not necessarily controlled by caveolin-1.

5. Conclusions

To conclude, our current study indicates that the mouse pulmonary endothelial β_2 -AR is coupled to a $G_{i/o}$ -Src kinase-PI3K/Akt pathway to promote eNOS phosphorylation at Ser¹¹⁷⁷ leading to a NO-dependent vasorelaxation, and that caveolin-1 exerts a negative control on this response, which is removed by its phosphorylation at Tyr¹⁴ through a $G_{i/o}$ -Src kinase pathway (Fig. 7). In addition, our data clearly show that the mouse pulmonary endothelial β_2 - and muscarinic-receptors activate eNOS through distinct signaling pathways, which differ especially in their dependence on caveolin-1. This further supports the concept that mechanisms underlying eNOS activation are key determinants of pulmonary endothelial dysfunction and may explain why, unlike the muscarinic relaxation, the β_2 -adrenergic relaxation is preserved in pulmonary arteries isolated from mice subjected to chronic hypoxia [6].

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Legends for figures

Figure 1: Role of $G_{i/o}$ proteins in the β_2 -AR-mediated relaxant response in mouse pulmonary artery. The relaxant responses to 0.1 μ M procaterol and 10 μ M acetylcholine were evaluated in arteries precontracted with $\text{PGF}_{2\alpha}$, which were pre-incubated in the absence or presence of PTX (400 ng/ml for 12 hours). **A: Representative traces of wall tension recording showing the relaxant effect of procaterol in pulmonary arteries pretreated in the absence (*Control*) or presence of PTX (+*PTX*).** **B: Relaxations induced by procaterol and acetylcholine** are expressed as a percentage of $\text{PGF}_{2\alpha}$ -induced contraction. Data are mean $\pm$ SEM of 8 experiments. * $P < 0.05$ versus control.

Figure 2: Role of PI3K/Akt pathway and Src-kinase in the β_2 -AR-mediated relaxant response in mouse pulmonary artery. The relaxant response to 0.1 μ M procaterol was evaluated in arteries precontracted with $\text{PGF}_{2\alpha}$, in the absence or presence of inhibitors of PI3K (0.1 μ M wortmannin or 10 μ M LY 294002), Akt (5 μ M Akt inhibitor X), Src-kinase (10 μ M PP2), or MAPK (10 μ M UO126, 20 μ M PD 98059, 5 μ M SB 203580 or 10 μ M SP 600125) or AMPK (2 μ M compound C). **A-C: Representative traces of wall tension recording showing the relaxant effect of procaterol in pulmonary arteries pretreated in the presence of vehicle (*DMSO*; A), wortmannin (*B*) and UO126 (*C*).** **D&E: Relaxations induced by procaterol in the absence (*procaterol*) or presence of the different kinase inhibitors (*D*: wortmannin, LY 294002, Akt inhibitor X or PP2; *E*: UO126, PD 98059, SB 203580, SP 600125, or compound C)** are expressed as a percentage of $\text{PGF}_{2\alpha}$ -induced contraction. Data are mean $\pm$ SEM of 3-7 experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus procaterol alone.

Figure 3: β_2 -AR-stimulation induces eNOS phosphorylation through $G_{i/o}$ proteins, PI3K/Akt and Src-kinase pathways in mouse pulmonary arteries. Western blot analysis for phospho-eNOS (Ser¹¹⁷⁷) and total eNOS proteins was performed on arteries incubated with 0.1 μ M procaterol (*Procaterol*) or not (*Control*). A: Effect of 30 sec and 5 min incubation times with procaterol. *Top*, representative blots. *Bottom*, densitometric ratios of phospho-eNOS (Ser¹¹⁷⁷)/eNOS bands, expressed as mean $\pm$ SEM of 3 experiments. $^{\S} P < 0.05$ versus control. B: Effect of a 5 min-incubation with procaterol in the absence or presence of Akt inhibitor X (5 μ M) or PP2 (10 μ M, a Src-kinase inhibitor) or in arteries pretreated or not with PTX (400 ng/ml for 12 hours). *Top*, representative blots. *Bottom*, densitometric ratios of phospho-eNOS (Ser¹¹⁷⁷)/eNOS bands are expressed as mean $\pm$ SEM of 3-5 experiments. $^{\S} P < 0.05$ versus control. $^* P < 0.05$ versus procaterol alone.

Figure 4: Role of membrane cholesterol in the β_2 -AR-mediated relaxant response in mouse pulmonary artery. The relaxant responses to 0.1 μ M procaterol and 10 μ M acetylcholine were evaluated in arteries precontracted with PGF_{2 α} , in the absence (*Control*) or presence of 1 mM methyl- β -cyclodextrin (+ methyl- β -cyclodextrin). A: Representative traces of wall tension recording showing the relaxant effect of procaterol in pulmonary arteries pretreated in the absence or presence of methyl- β -cyclodextrin. B: Relaxations induced by procaterol and acetylcholine are expressed as a percentage of PGF_{2 α} -induced contraction. Data are mean $\pm$ SEM of 4 experiments. $^* P < 0.05$ versus control.

Figure 5: Role of caveolin-1 in the β_2 -AR-mediated relaxant response in mouse pulmonary arteries.

A: Expression of caveolin-1 protein (*Cav-1*) assessed by Western blot in homogenates from pulmonary arteries isolated from WT and Cav-1 KO mice. Blots shown are representative of 3 experiments. β -actin was used as a loading control.

B: Representative transmission electron micrographs performed in pulmonary arteries from WT and Cav-1 KO mice. Arrows indicate the presence of caveolae (defined as 50-100 nm round-shaped membrane invaginations) at the endothelial cell membrane. Original magnification at x 60 000 (with further magnification in insets). Photos are representative of experiments performed on 3 WT and 3 Cav-1 KO mice.

C: Representative trace of wall tension recording in pulmonary arteries isolated from WT and Cav-1 KO mice showing the relaxant effect of 0.1 μ M procaterol after PGF_{2 α} -induced contraction. The hatched area corresponds to the area of the relaxant effect of procaterol which is quantified in panel D.

D: Area of the relaxant effect of 0.1 μ M procaterol (hatched area in panel C) and 10 μ M acetylcholine in pulmonary arteries isolated from WT and Cav-1 KO mice and precontracted with PGF_{2 α} . Results (in arbitrary units, a.u.) are expressed as area of relaxation reported to the amplitude of contraction induced by PGF_{2 α} . Data are mean $\pm$ SEM of 6 to 8 experiments. [#] $P < 0.05$ versus WT mice.

E: Relaxant effect to 0.1 μ M procaterol and 10 μ M acetylcholine in pulmonary arteries isolated from WT and Cav-1 KO mice, in the absence (*Control*) or presence (+ *L-NAME*) of 300 μ M L-NAME. Results are expressed as a percentage of PGF_{2 α} -induced contraction. Data are mean $\pm$ SEM of 4 to 8 experiments. *** $P < 0.001$ versus control. [#] $P < 0.05$ versus WT mice.

Figure 6: Role of G_{i/o} proteins and Src-kinase pathway in the β_2 -AR-mediated phosphorylation of caveolin-1 at Tyr¹⁴ in mouse pulmonary arteries. Western blot analysis for phospho-caveolin-1 (Tyr¹⁴) and total caveolin-1 proteins was performed in control arteries

(*Control*) and arteries incubated with 0.1 μ M procaterol for 30 sec (*Procaterol*) in the absence or presence of PP2 (10 μ M) or arteries pretreated with PTX (400 ng/ml for 12 hours). *Top*, representative blots. *Bottom*, densitometric ratios of phospho-caveolin-1 (Tyr¹⁴)/caveolin-1 bands are expressed as mean $\pm$ SEM of 4 experiments. ^{§§§} $P < 0.001$ versus control. ^{*} $P < 0.05$ versus procaterol alone.

Figure 7: Schematic representation of the proposed signaling pathway responsible for eNOS-dependent β_2 -AR-mediated vasorelaxation in mouse pulmonary artery. *Left*, In endothelial cell, caveolin-1 (*Cav-1*) maintains eNOS in its inactive state. *Right*, Upon β_2 -AR stimulation, the activated G_{i/o}/Src-kinase (*Src K.*) pathway elicits eNOS activation through two pathways: a direct phosphorylation of caveolin-1 at Tyr¹⁴ which dissociates eNOS from caveolin-1, and an indirect phosphorylation of eNOS at Ser¹¹⁷⁷ through a PI3K/Akt pathway. NO produced by activated eNOS induces relaxation of the smooth muscle cell, through a guanylate cyclase (GC)-cGMP-dependent pathway [6]. ERK1/2, p38MAPK, JNK and AMPK are not involved in the β_2 -adrenergic vasorelaxation. Note that the exact membrane localization of the β_2 -AR, in or outside the caveolae, remains unknown. Pharmacological agents used in experiments to inhibit the activity of different enzymes of the pathway are indicated in blue boxes.