BBA - Molecular Cell Research 1867 (2020) 118848

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Lysophosphatidic acid receptor 5 transactivation of TGFBR1 stimulates the mRNA expression of proteoglycan synthesizing genes *XYLT1* and *CHST3*

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ARTICLE INFO

Keywords:
G protein-coupled receptor
Kinase receptors
Smads
Vascular smooth muscle cells
Biglycan
TGF-beta receptor

ABSTRACT

Lysophosphatidic acid (LPA) via transactivation dependent signalling pathways contributes to a plethora of physiological and pathophysiological responses. In the vasculature, hyperelongation of glycosaminoglycan (GAG) chains on proteoglycans leads to lipid retention in the intima resulting in the early pathogenesis of atherosclerosis. Therefore, we investigated and defined the contribution of transactivation dependent signalling in LPA mediated GAG chain hyperelongation in human vascular smooth muscle cells (VSMCs).

LPA acting via the LPA receptor 5 (LPAR5) transactivates the TGFBR1 to stimulate the mRNA expression of GAG initiation and elongation genes xylosyltransferase-1 (*XYLT1*) and chondroitin 6-sulfotransferase-1 (*CHST3*), respectively. We found that LPA stimulates ROS and Akt signalling in VSMCs, however they are not associated in LPAR5 transactivation of the TGFBR1. We observed that LPA via ROCK dependent pathways transactivates the TGFBR1 to stimulate genes associated with GAG chain elongation. We demonstrate that GPCR transactivation of the TGFBR1 occurs via a universal biochemical mechanism and the identified effectors represent potential therapeutic targets to inhibit pathophysiological effects of GPCR transactivation of the TGFBR1.

1. Introduction

Lysophosphatidic acid (LPA) is a collective term for a series of endogenous signalling molecules that comprise of a glycerol backbone, a phosphate head group and a single tail of acyl chain with varied length and saturation [1,2]. LPA signals via six well recognised LPA receptors (LPARs) (LPAR1-LPAR6) [3], which are members of the G protein-coupled receptor (GPCR) family. GPCRs transduce their signal via three well established paradigms [4]. Transactivation independent signalling that employs heterotrimeric G proteins to initiate downstream cascades [5], β-arrestin dependent signalling pathways [6] or via transactivation dependent signalling [7]. In this paradigm, the activation of one GPCR promptly activates a secondary receptor to trigger diverse downstream responses [8]. The GPCR transactivation dependent signalling has been extended to include serine/theronine kinase receptor (S/TKR) transactivation in addition to the long established protein tyrosin kinase receptor (PTKR) transactivation [4,9] examplified by transforming growth factor receptor 1 (TGFBR1) and epidermal growth factor receptor (EGFR) transactivation, respectively [10–14].

Atherosclerosis commences with the accumulation of low density lipoproteins (LDLs) in the vessel wall by modified proteoglycans with hyperelongated glycosaminoglycan (GAG) chains [15–17]. The hyperelongation of GAG chains on proteoglycans is modulated by many growth factors [18–20]. In experimental models of atherosclerosis, such as high fat fed genetically modified mice, the administration of compounds which block growth factor mediated GAG chain hyperelongation and binding of LDL-cholesterol in vitro, also block the deposition of lipids in the vessel wall [19,20]. Therefore, blocking GAG chain hyperelongation by inhibiting the relevant growth factor mediated signalling pathways is a valid strategy to prevent atherosclerosis. The initiation and hyperelongation of GAG chains involves multiple enzymes [21–23], including the initiation xylosyltransferase-1 (*XYLT1*), elongation and sulfation enzymes chondroitin-4-O-sulfotransferase-1 (*CHST11*) as well as chondroitin-6-O-sulfotransferase-1 (*CHST3*). Therefore, characterising the underlying signalling pathways that control the expression of these enzymes and their genes will contribute to our understanding of the mechanisms of atherosclerosis.

In human vascular smooth muscle cells (VSMCs), we have previously observed and reported the effects of EGF [24], platelet-derived growth factor (PDGF) [20,25] and transforming growth factor beta 1

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https://doi.org/10.1016/j.bbamcr.2020.118848
Received 5 August 2020; Received in revised form 30 August 2020; Accepted 3 September 2020
Available online 11 September 2020
0167-4889/

(TGF-β1) [26,27] on GAG chain hyperelongation. In the light of the transactivation dependent signalling concept, we have reported that GPCR ligand endothelin-1 [11] stimulates GAG chain hyperelongation via the endothelin receptor A mediated transactivation of TGFBR1. Thrombin via its protease-activated receptor-1 (PAR-1) transactivates both the EGFR and TGFBR1 to regulate GAG chain hyperelongation [28] and the mRNA expression of GAG chain synthesizing genes [12]. These findings suggest transactivation signalling pathway is playing a critical role in regulating GAG chain hyperelongation.

Transactivation dependent signalling is playing a vital role in the vascular system, such as regulating the above mentioned GAG chain hyperelongation [11,28]. However, in human VSMCs, this paradigm of LPA signalling has not yet been reported as well as the role of LPA on GAG chain hyperelongation. In this paper, we addressed these questions by investigating LPA transactivation of kinase receptors and its role on GAG chain synthesizing gene expression.

2. Materials and methods

2.1. Materials

Human aortic VSMCs: T/G HA-VSMC (ATCC® CRL-1999™) and TC LPA5 4 were purchased from In Vitro Technologies Life science (VIC, AUS). Ham's F-12 K (Kaighn's) medium, antibiotics solution (10,000 U/mL penicillin, 10,000 μg/mL streptomycin), GlutaMAX™-I (100×), 0.25% trypsin-EDTA (1×), fetal bovine serum (FBS), Pierce™ bicinchoninic acid protein assay kit and chemiluminescent molecular weight marker (MagicMark XP), Invitrogen™ 50 bp DNA ladder, SYBR Gold™ nucleic acid gel stain and Fluo-4 AM were purchased from Thermo Fisher Scientific (VIC, AUS). TGF-β1, EGF, phospho-Smad2 (Ser465/467) (3108S), phospho-Erk (Thr202/Tyr204) (4377S), phospho-Akt (Ser473) (4058S), phospho-Ezrin (Thr567)/Radixin (Thr567)/Moesin (Thr558) (3141S), GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase) (3683S) and rabbit IgG HRP-linked antibody (7074S) were purchased from Australian Biosearch (WA, AUS). LPA receptor control cell lysates and antibodies to LPAR3 and LPAR5 were purchased from Abcam (VIC, AUS). LPAR1/2/4/6 antibodies were purchased from Alomone Labs (Jerusalem, Israel). Bovine serum albumin (BSA) was from Bovogen Biologicals Pty Ltd. (VIC, AUS). LPA (CAS: 22556-62-3), SB431542, AG1478, AM095, H2L5186303, diphenyleneiodonium chloride (DPI), N-Acetyl-Cysteine (NAC), 2′,7′-Dichlorofluorescence diacetate (H_2DCF-DA) dye and endothelial cell growth supplement (ECGS) were purchased from Sigma Aldrich (NSW, AUS). Certified low range ultra-agarose, Bio-Rad Trans-Blot® Turbo Turbo RTA transfer kit, polyvinylidene fluoride (PVDF) membrane, 30% acrylamide/bis-acrylamide solution, ammonium persulfate (APS) and Image Lab software were from BioRad Laboratories (VIC, AUS). Primers for *RRN18S*, *LPARs*, *XYLT1* and *CHST3*; RNeasy® Mini Kit, QuantiTect® Reverse Transcription Kit and QuantiNova™ SYBR® Green PCR Kit were purchased from Qiagen (VIC, AUS).

2.2. Cell culture

Human aortic vascular smooth muscle cells (VSMCs) were maintained in complete Ham's F-12K medium (10% FBS and 1% antibiotics, 5% GlutaMAX, 0.3 mg/mL ECGS) at 37 °C with 5% CO_2. VSMCs were seeded in 60 mm dishes or 96-well plates for experiments. Cells were grown to confluence, then rendered quiescent using F-12K medium (0.1% FBS and 1% antibiotics) for 48 h prior treatments. Treatment details are given in the figure legends.

2.3. Western blotting

Whole cell lysates were separated on 10% or 12% SDS-PAGE and semi-dry transferred onto PVDF membranes. Membranes were blocked with 5% BSA or 5% skim milk, then incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. Blots were imaged using the Bio-Rad gel documentation system and densitometry analysis was performed with Image Lab 5.2.1.

2.4. mRNA expression

The mRNA expression of genes was determined by quantitative real time polymerase chain reaction (qRT-PCR). Total RNA was isolated from treated VSMCs with RNeasy Mini kit according to the instructions of the manufacturer. RNA concentration and purity were assessed by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). 1000 ng RNA was used to synthesize first strand cDNA using Quantitect reverse transcriptase kit. qRT-PCR was performed using Qiagen Rotor Gene Q and QuantiNova SYBR green PCR master mix kit. Data was normalised to the ribosomal *18S* housekeeping gene to control variations between individual experiments. Relative expression of mRNA levels was quantified using comparative delta delta Ct method.

2.5. Agarose gel electrophoresis

To visualise the PCR product, 20 μL amplified DNA solution was separated on a 1.5% agarose gel in TEA buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.3) parallel with 8 μL DNA ladder. After separation, the gel was then incubated with 0.01% SYBR Gold for 30 min, followed by image capture using the Bio-Rad gel documentation system.

2.6. Intracellular calcium assay

Intracellular calcium was quantified by fluorescence assay using Fluo-4 AM. VSMCs were seeded into a 96 well black plate with a density of 10,000 cells/well. Cells were rendered quiescent in Ham's F-12K (0.1% FBS and 1% antibiotics) for 48 h. Krebs-HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM glucose, 2 mM probenecid, pH 7.4) was used to wash the cells prior to incubation with 4 μM Fluo-4 AM for 30 min at 37 °C and 5% CO_2. The dye was then removed and VSMCs were washed twice with Krebs-HEPES buffer and incubated with antagonists for 15 min. Agonists were injected into 96-well plate using FLIPR Tetra® High-Throughput Cellular Screening System. Fluorescence intensity was measured at excitation/emission of 494/506 nm. The fluorescence intensity was used to calculate the fold change of intracellular calcium.

2.7. Intracellular ROS assay

VSMCs seeded in a 96-well plate at 10,000 cells per well were rendered quiescent for 48 h in F-12K (0.1% FBS and 1% antibiotics). Then, cells were washed with 100 μl of Krebs-HEPES buffer [29] and incubated with 20 μM H_2DCF-DA for 45 min at 37 °C and 5% CO_2. The dye was gently removed and replaced with Krebs-HEPES buffer in the presence of LPA. The fluorescence was measured using Ensight™ Multimode Plate Reader at excitation/emission of 485/535 nm. The fluorescence intensity was used to calculate the fold change of intracellular ROS level.

2.8. Statistical analysis

Normalised data is expressed as the mean ± standard error of the mean (SEM) of four independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference *post-hoc* analysis. Results were considered significant when the probability was less than 0.05 ($*p < 0.05$) and 0.01 ($**p < 0.01$).

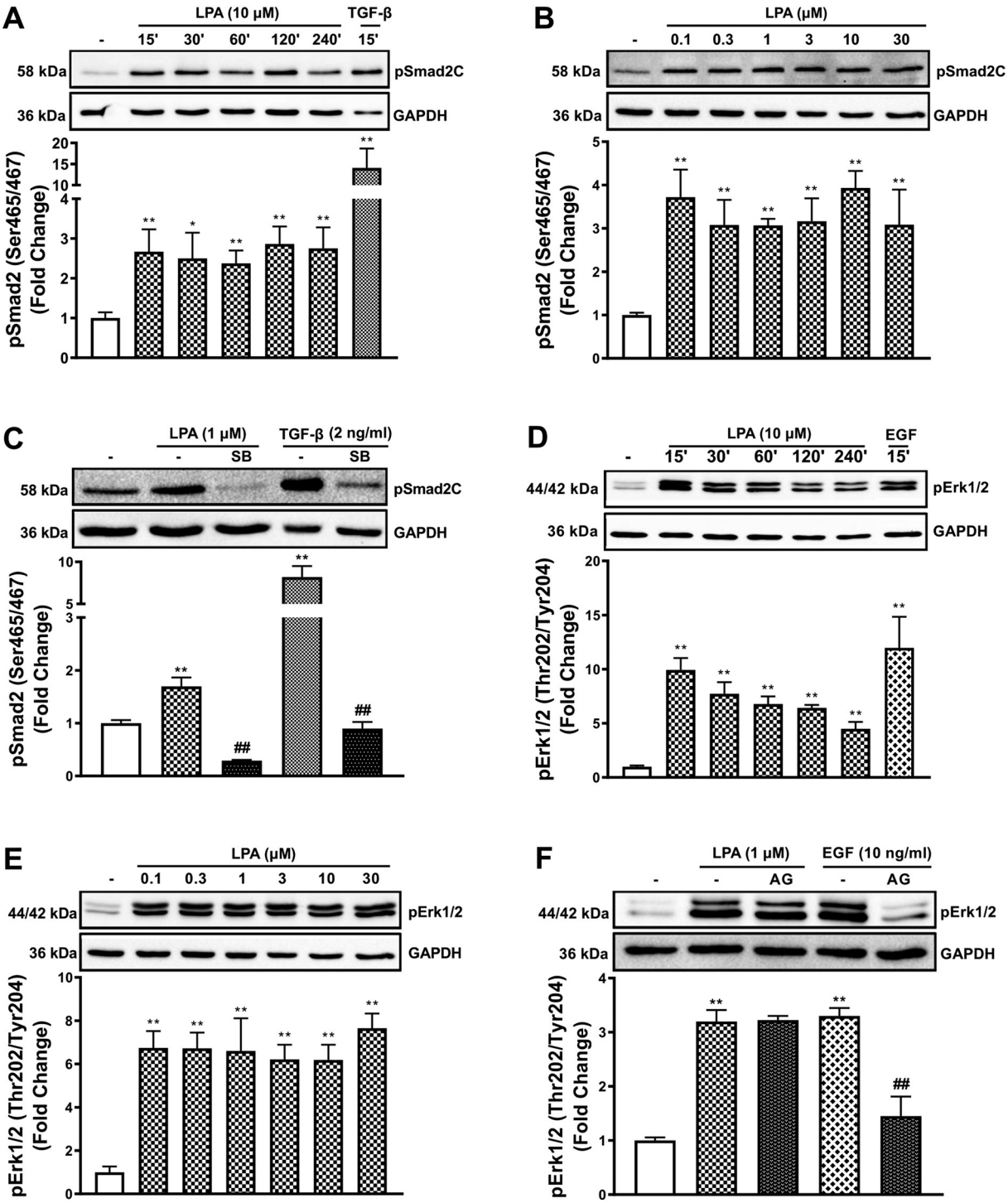

Fig. 1. LPA transactivation of kinase receptors. (A, D) Human VSMCs were treated with LPA (10 μM) over 240 min time course or (A) TGF-β (2 ng/mL), (D) EGF (10 ng/mL) for 15 min. (B, E) Human VSMCs were treated with LPA (0.1 μM–30 μM) for 15 min. (C, F) Human VSMCs were treated with LPA (1 μM) for 15 min in the presence and absence of (C) SB431542 (3 μM) or (F) AG1478 (5 μM). Blots were probed with antibodies to phospho-Smad2 (Ser465/467) (1:1000) or phospho-Erk1/2 (Thr202/Tyr204) (1:4000) and secondary anti-rabbit IgG antibody (1:2000). Blots are representative of four-independent experiments. Blot density was normalised to GAPDH to offset the effect of unequal sample loading. Panels B and E share the same GAPDH. Histogram represents band density expressed as fold per basal (presented as mean ± SEM). Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. *, $p < 0.05$ and **, $p < 0.01$ versus basal; ##, $p < 0.01$ versus corresponding agonists treated samples.

3. Results

3.1. LPA induces TGFBR1 but not EGFR transactivation in human VSMCs

LPA has been reported to transactivate TGFBR1 in human lung epithelial cells [30] and in human airway smooth muscle cells [31]. To assess LPA mediated TGFBR1 transactivation in human VSMCs, we measured Smad2 carboxyl-terminal phosphorylation (pSmad2C) from cells time dependently treated with LPA (10 μM) (Fig. 1A). LPA treated VSMCs showed a rapid increase of pSmad2C with a 2.7-fold ($p < 0.01$)

change observed at 15 min and this was maintained over the 240 min treatment period. TGF-β1 (2 ng/mL) treatment used as a positive control increased the pSmad2C to 14-fold ($p < 0.01$). A dose response study of LPA (0.1 μM–30 μM) on the pSmad2C (Fig. 1B). VSMCs treated with 0.1 μM LPA resulted in a 3.7-fold ($p < 0.01$) increase in pSmad2C and this response was sustained under all treatment conditions.

To characterise whether LPA stimulated pSmad2C is occurring via transactivation dependent mechanisms, we utilised the well-characterized TGFBR1 kinase inhibitor, SB431542 [32] and measured LPA mediated pSmad2C (Fig. 1C). Treatment of VSMCs with LPA (1 μM) for 15 min stimulated a 1.7-fold ($p < 0.01$) increase in pSmad2C that was completely abolished by TGFBR1 antagonist, SB431542. Treatment of VSMCs with TGF-β1 in the presence and absence of SB431542 served as controls. TGF-β1 treatment stimulated an 8.2-fold ($p < 0.01$) increase in pSmad2C which was completely blocked by SB431542. These results demonstrate that LPA stimulates the phosphorylation of the Smad2 carboxy terminal via transactivation of TGFBR1.

LPA transactivates EGFR to regulate DNA synthesis and growth factor expression in rat-1 fibroblasts [7] and human umbilical vein endothelial cells [33]. To investigate whether LPA can transactivate EGFR in VSMCs, time and dose dependent experiment were conducted (Fig. 1D and E) and the downstream product of EGFR, phosphorylated Erk1/2 (pErk1/2), was measured. VSMCs treated with LPA (10 μM) showed a rapid 10-fold ($p < 0.01$) increase of pErk1/2 (Fig. 1D). LPA stimulated pErk1/2 was sustained and gradually declined to 4.5-fold ($p < 0.01$) stimulation at 240 min treatment. Treatment with EGF was used as a positive control and as expected EGF stimulated a large ($p < 0.01$) increase in pErk1/2. This was followed by a dose dependent evaluation of LPA (0.1 μM–30 μM) mediated pErk1/2 (Fig. 1E). LPA treatment of VSMCs stimulated pErk1/2 to 6.7-fold ($p < 0.01$) at 0.1 μM and this response was sustained across all the range of LPA concentrations. To evaluate whether LPA mediated pErk1/2 was occurring via transactivation of the EGFR, we utilised specific EGFR antagonist, AG1478 [34] (Fig. 1F). Treatment of VSMCs with LPA (1 μM) increased pErk1/2 to 3.2-fold ($p < 0.01$); however, this response was unaffected in the presence of AG1478. Treatment with EGF in the presence and absence of AG1478 served as controls. EGF treatment stimulated pErk1/2 to 3.3-fold ($p < 0.01$) and this response was blocked by AG1478, validating AG1478 as an EGFR antagonist. This data demonstrates that LPA mediated phosphorylation of Erk1/2 was not occurring via transactivation of the EGFR. Taken together, the data demonstrates that in VSMCs, LPA transactivates the TGFBR1 but not the EGFR.

3.2. LPA mediated TGFBR1 transactivation is occurring via LPAR5

LPA can signal via at least six known GPCRs (LPAR1-6) which are differentially expressed in human tissues [3]. We sought to characterise the LPARs that are expressed in human VSMCs with the assessment of the gene expression and protein expression. At the mRNA level (Fig. 2A), LPAR1 was the most abundantly expressed receptor in human VSMCs. LPAR2 and LPAR6 were the next highly expressed receptors. LPAR4, LPAR5 were less expressed while LPAR3 was not expressed in VSMCs. To visualise the results, PCR products were subjected to agarose gel electrophoresis which correlates with the quantitative data in the histogram. At the protein level (Fig. 2B), the LPAR1 was the most abundantly expressed receptor in human VSMCs, with LPAR2 and LPAR5 also detected. In contrast, the LPAR3, LPAR4 and LPAR6 were not detected. Therefore, LPAR1, LPAR2 and LPAR5 are expressed in human VSMCs and they are the subjects of future functional studies.

To characterise the functionality of LPAR1, LPAR2 and LPAR5, we utilised specific receptor antagonists and measured the downstream product of LPARs, intracellular calcium release (Fig. 2C, D, E). Treatment with LPA (10 μM) induced a rapid increase in intracellular calcium to 8-fold ($p < 0.01$) and in the presence of LPAR1 antagonist, AM095, this response was dose dependently inhibited with a complete inhibition observed at 10 μM (Fig. 2C). In the presence of the LPAR2 inhibitor, H2L5186303, LPA stimulated intracellular calcium was unaffected under all treatment conditions. Gαq protein inhibitor, YM-254890, served as a control and inhibited LPA stimulated intracellular calcium (Fig. 2D). In the presence of the LPAR5 antagonist, TC LPAR5 4, LPA mediated intracellular calcium was dose dependently inhibited with a complete inhibition observed at 10 μM (Fig. 2E). This data demonstrates that although LPAR1, LPAR2 and LPAR5 are abundantly expressed in human VSMCs, only LPAR1 and LPAR5 are biologically functional in these cells.

To characterise the LPAR(s) involved in the LPA transactivation of the TGFBR1, pharmacological inhibitors to LPAR1 (AM095), LPAR2 (H2L5186303) and LPAR5 (TC LPA5 4) were utilised (Fig. 2F). VSMCs were pre-incubated with LPAR inhibitors for 30 min followed with LPA (10 μM) for 30 min. LPA treated cells resulted in a 3.2-fold ($p < 0.01$) stimulation of pSmad2C. In the presence of LPAR1 inhibitor AM095 and LPAR2 inhibitor H2L5186303, there was no observed change of pSmad2C. However, in the presence of the LPAR5 inhibitor, the LPA mediated pSmad2C was blocked by 80%, indicating LPA mediated TGFBR1 transactivation occurs via LPAR5.

3.3. LPAR5 mediated TGFBR1 transactivation is not regulated by Akt signalling

Most TGFBRs reside in the intracellular compartments of the cells. TGFBR1 mobilization to the cell surface is driven by Akt mediated phosphorylation of the membrane associated Rab-GTPase activating protein, AS160 [35,36], resulting in increased sensitivity to endogenous or exogenous TGF-β. Therefore, we sought to investigate whether LPA stimulates Akt in human VSMCs and whether Akt was driving the LPA transactivation of the TGFBR1. VSMCs were treated with LPA in the presence and absence of two Akt inhibitors, allosteric inhibitor MK-2206 (1 μM) [37] and ATP competitive inhibitor GSK690693 (1 μM) [38] (Fig. 3A). Treatment with LPA stimulated a 4.4-fold ($p < 0.01$) increase in the phosphorylation of Akt which was abolished by MK-2206 but not GSK690693. Mechanistically, GSK690693 constrains Akt dephosphorylation by stabilizing a conformation where phosphorylated sites on Akt are inaccessible to phosphatases [38]. Therefore, GSK690693 enhances phospho-Akt, however inhibits downstream effectors of Akt [39], including phospho-AS160 (data not shown).

To investigate whether LPA mediated phosphorylation of Akt sensitizes the cells to TGFBR1 transactivation, we evaluated whether LPA mediated pSmad2C was regulated by Akt dependent pathways (Fig. 3B). Treatment with LPA stimulated pSmad2C to 2.2-fold ($p < 0.05$) which was unaffected in the presence of Akt inhibitors MK-2206 and GSK690693, whereas LPA stimulated pSmad2C was inhibited by TGFBR1 antagonist, SB431542 (Fig. 3B). This data demonstrates that treatment of VSMCs with LPA can stimulate the phosphorylation of Akt. However, LPA mediated transactivation of the TGFBR1 is not regulated by Akt dependent signalling pathways.

3.4. LPAR5 mediated TGFBR1 transactivation is intermediated by ROCK signalling

Several biochemical mechanisms exist for TGFBR1 activation including proteolytic activation of TGFBR1, ROCK/integrin dependent signalling, and activation by reactive oxygen species (ROS) [40,41]. We have previously demonstrated that thrombin transactivation of the TGFBR1 occurs via ROCK dependent signalling pathways [28,42]. We have recently identified that in VSMCs endogenous ROS activates the TGFBR1 and downstream pSmad2C [29,43]. We have also shown that GPCR transactivation of the TGFBR1 is not dependent on MMPs and the proteolytic activation of the TGFBR1 [28,42]. Therefore, our aim was to characterise the mechanisms of LPA mediated transactivation of the TGFBR1.

LPA can stimulate ROS production in different cellular context

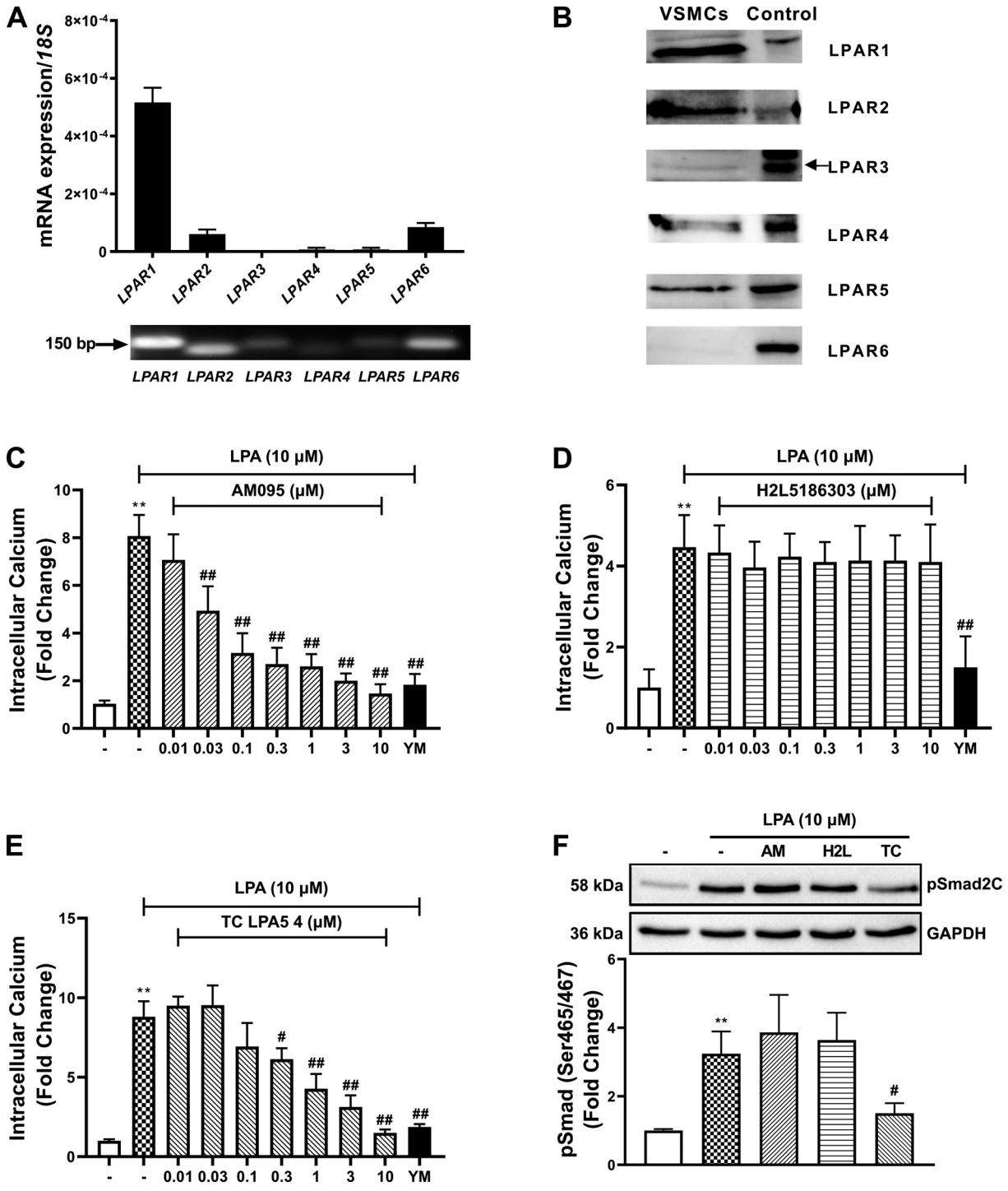

Fig. 2. The expression and biological function of LPAR(s) in human VSMCs. (A). The mRNA expression of individual receptors was normalised to the expression of *18S* and expressed as mean ± SEM ($n = 3$). Samples were then resolved on agarose gel for visualisation. (B) Lysates from VSMCs were resolved on SDS-PAGE with the following lysates used as positive controls: A549 (4 μg) (LPAR1), HL60 (10 μg) (LPAR2), HepG2 (20 μg) (LPAR3), HepG2 (5 μg) (LPAR4), HT29 (20 μg) (LPAR5) and mouse heart tissue (10 μg) (LPAR6). Transferred proteins were blotted with anti-LPAR1 (1:400) or with anti-LPAR (2, 3, 4, 5, 6) (1:200) followed with secondary anti-rabbit IgG (1:2000). To measure functionality of expressed LPARs, intracellular calcium was measured in the presence of receptor antagonists (C) LPAR1: AM095 (D) LPAR2: H2L5186303 and (E) LPAR5: TC LPA5 4; Gαq inhibitor YM-254890 (10 μM) served as a control. Histogram represents fluorescence intensity fold change compared with basal level. Results are expressed as mean ± SEM (n = 3). (F) VSMCs were pre-incubated with receptor antagonists LPAR1 AM095 (10 μM), LPAR2 H2L5186303 (10 μM) or LPAR5 TC LPA5 4 (10 μM) followed with LPA (10 μM) for 30 min. Blots were probed anti-phospho-Smad2 (Ser465/467) (1:1000) and secondary anti-rabbit IgG (1:2000). Blots are representative of four-independent experiments. Histogram represents band density expressed as fold per basal (presented as mean ± SEM). Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. **, $p < 0.01$ versus basal; #, $p < 0.05$ and ##, $p < 0.01$ versus LPA treated samples.

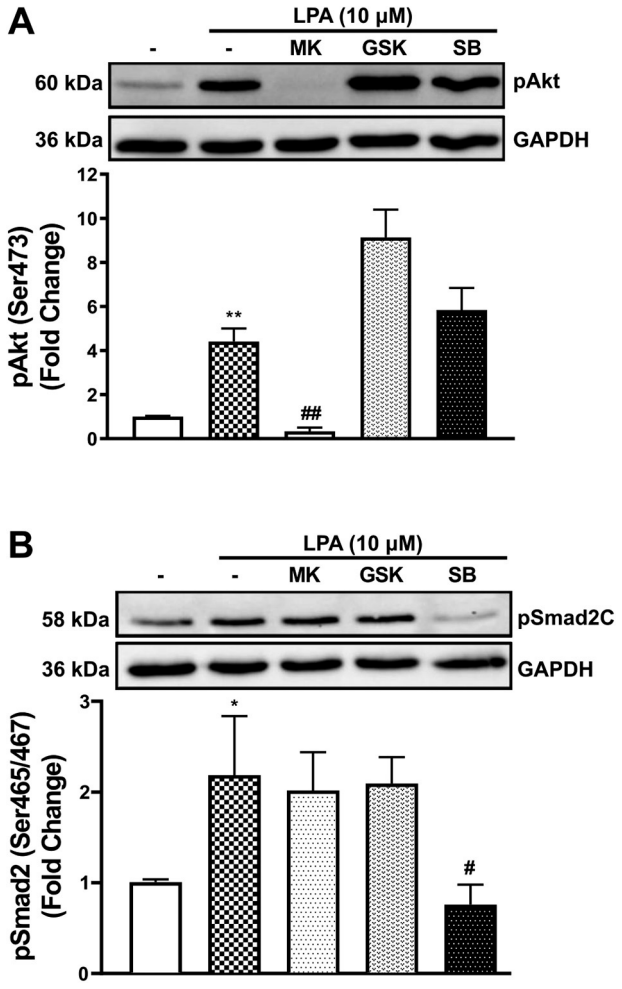

Fig. 3. The role of Akt signalling in LPAR5 transactivation of TGFBR1. VSMCs were pre-incubated with MK-2206 (1 μM), GSK690693 (1 μM) or SB431542 (3 μM) for 30 min prior the treatment of LPA (10 μM) for another 30 min. Blots were probed with antibodies to (A) phospho-Akt (Ser473) (1:1000) or (B) phospho-Smad2 (Ser465/467) (1:1000) and secondary anti-rabbit IgG (1:2000). Blots are representative of four-independent experiments. Histogram represents band density expressed as fold per basal (presented as mean ± SEM) after normalising with GAPDH. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. *, $p < 0.05$ and **, $p < 0.01$ versus basal; #, $p < 0.05$ and ##, $p < 0.01$ versus LPA treated sample.

[44,45], including the cells in the vasculature [46,47]. We examined the profile of LPA stimulated ROS generation in VSMCs. Cells were treated with LPA (10 μM) over a 120 min period (Fig. 4A). Upon treatment with LPA, VSMCs showed a rapid ROS production of 1.5-fold within 5 min compared to control and this response was maintained for up to 30 min. Treatment with LPA dose dependently increased intracellular ROS levels with a 1.5-fold ($p < 0.01$) stimulation observed at 10 μM LPA (Fig. 4B). These data demonstrate that in VSMCs treatment with LPA can stimulate intracellular ROS levels. Next, we examined the role of ROS in LPA transactivation response utilising a broad-spectrum NOX inhibitor, DPI and ROS scavenger, NAC. LPA treatment stimulated a 1.8-fold ($p < 0.01$) increase in pSmad2C. In the presence of DPI and NAC, LPA mediated pSmad2C was unaffected, however as expected TGFBR1 antagonist SB431542 completely abolished this response. Treatments with TGF-β in the presence and absence of the NOX/ROS antagonists served as controls. As expected, treatment with DPI and NAC had no effect on direct TGF-β mediated pSmad2C. These data demonstrate that although LPA can stimulate intracellular levels of ROS, LPA transactivation of the TGFBR1 is not dependent on NOX/ROS pathways.

To investigate whether LPA transactivation of the TGFBR1 is occurring via ROCK dependent pathways, we measured the immediate downstream product of ROCK, phosphorylated ezrin/radixin/moesin (pERM) (Fig. 4D). VSMCs treated with LPA (10 μM) stimulated a 2-fold ($p < 0.05$) increase in pERM within 15 min and a maximal stimulation of 2.2-fold ($p < 0.01$) observed at 30 min. We next utilised ROCK inhibitor Y27632 (10 μM) and observed that LPA mediated pSmad2C was completely abolished by both Y27632 and SB431542, indicating LPAR5 mediated TGFBR1 transactivation was ROCK dependent (Fig. 4E).

3.5. LPA stimulates the expression of GAG chain synthesizing genes

LPA is critical in multiple stages of the pathogenesis of atherosclerosis [2]. We wished to investigate the role of LPA in the early lipid binding phase of atherogenesis. Therefore, we studied LPA mediated GAG chain elongation as an early marker of vascular change and potentially lipid retention. GAG chain synthesis requires the combined action of multiple GAG synthesizing enzymes [21]. The initiation of the GAG chains is attributable to the expression of the *XYLT1* and the elongation and sulfation of the GAG chains correlates with *CHST3* expression. We measured the mRNA expression of these genes to assess the role of LPA on GAG chain initiation and elongation.

VSMCs were treated with LPA (10 μM) over 8 h or control TGF-β1 (2 ng/mL) for 4 h (Fig. 5). LPA time-dependently stimulated mRNA expression of *XYLT1* (Fig. 5A) with a 2.6-fold ($p < 0.01$) change observed at 6 h which then peaked at 8 h (3.0-fold, $p < 0.01$). TGF-β1 stimulated mRNA expression of *XYLT1* to 4.9-fold ($p < 0.01$). In contrast, LPA treatment resulted in the rapid increase in the mRNA expression of *CHST3* with 2.0-fold ($p < 0.05$) change observed at 2 h, and this stimulation was sustained for 8 h (Fig. 5B). As expected, treatment with TGF-β1 resulted in a 2.2-fold ($p < 0.05$) stimulation of *CHST3* expression. These results demonstrate that LPA can drive the mRNA expression of the genes involved in the initiation and elongation of the GAG chains.

3.6. LPA stimulated GAG chain synthesizing gene expression is regulated by LPAR1 and LPAR5

To characterise the LPAR(s) that drive the mRNA expression of GAG chain synthesizing genes, we pre-incubated the cells with pharmacological antagonists to LPAR1, AM095 (10 μM), LPAR2, H2L5186303 (10 μM) and LPAR5, TC LPA5 4 (10 μM) followed with LPA (10 μM) for 6 h. LPA treatment stimulated a 2.5-fold ($p < 0.01$) change of *XYLT1* expression (Fig. 6A), which was inhibited by 80% ($p < 0.05$) in the presence of LPAR1 inhibitor and was completely blocked by LPAR5 inhibitor; however, the LPAR2 inhibitor showed no effect. LPA treatment stimulated a 2.2-fold ($p < 0.01$) change of *CHST3* expression (Fig. 6B), which was partially mitigated by 50% and 60% in the presence of LPAR1 inhibitor and LPAR5 inhibitor, respectively. The LPAR2 inhibitor had no functional role on LPA mediated *CHST3* expression. The data shows LPAR1 and LPAR5 but not the LPAR2 regulate LPA mediated GAG chain synthesizing gene expression.

3.7. LPAR5 stimulated mRNA expression of GAG chain synthesizing gene XYLT1 and CHST3 is regulated by TGFBR1 transactivation pathway

Thrombin transactivates both EGFR and TGFBR1 to elicit GAG chain hyperelongation [28] and GAG chain synthesizing gene expression [12]. To examine the contribution of transactivation dependent pathways in LPA mediated GAG chain synthesizing gene expression, we utilised specific TGFBR1 inhibitor, SB431542 (3 μM) and EGFR antagonist, AG1478 (5 μM). LPA treatment stimulated a 2.8-fold ($p < 0.01$) change of *XYLT1* expression (Fig. 7A), which was inhibited by 90% ($p < 0.01$) in the presence of SB431542 and unaffected in the

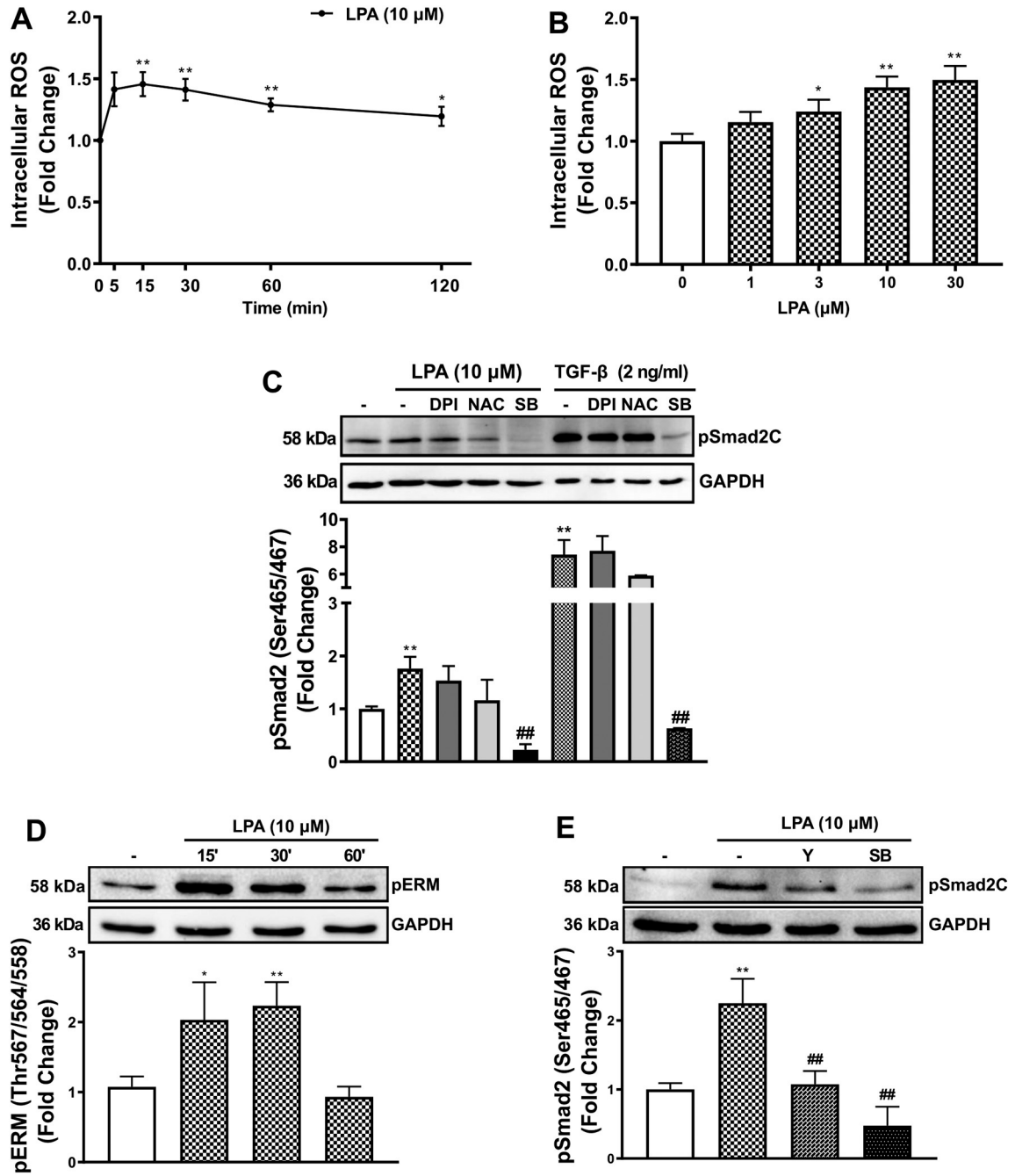

Fig. 4. The mechanistic involved in LPAR5 mediated transactivation of the TGFBR1. VSMCs were treated with (A) LPA (10 μM) for 5–120 min or (B) LPA (1–30 μM) for 15 min. Histograms represent fluorescence intensity expressed as fold per basal of intracellular ROS production. VSMCs were treated with (C) LPA (10 μM) or TGF-β (2 ng/ml) in the presence and absence of NOX inhibitor DPI (10 μM), anti-oxidant NAC (5 mM) or TGFBR1 antagonist SB43152 (3 μM) (D) LPA (10 μM) 0–60 min or with (E) LPA (10 μM) in the presence of ROCK inhibitor Y27632 (10 μM) or SB4315412 (3 μM). Blots were probed with (C and E) anti-phospho-Smad2 (Ser465/467) (1:1000) or (D) anti-phospho-ERM (Thr567/564/558) (1:1000) and secondary anti-rabbit IgG (1:2000). Blots are representative of three to four independent experiments. Histogram represents band density expressed as fold per basal (presented as mean ± SEM) after normalising with GAPDH. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. *, $p < 0.05$ and **, $p < 0.01$ versus basal; ##, $p < 0.01$ versus agonist.

presence of AG1478. The treatment with LPA induced the mRNA expression of *CHST3* to 2.4-fold ($p < 0.01$) change (Fig. 7B) that was partially blocked by SB431542 ($p < 0.05$), whereas AG1478 showed no inhibition. The data together demonstrates that LPA transactivates TGFBR1 but not EGFR to mediate the expression of GAG chain biosynthesizing genes.

4. Discussion

LPA is involved in multiple aspects of the pathogenesis of atherosclerosis, such as promoting inflammation, platelet aggregation, endothelial permeability, VSMC dedifferentiation and extracellular matrix remodelling [2]. Modified GAG chains in the intima bind and retain LDL-cholesterol as the initiating step of atherosclerosis [15–17]. In this study, we investigated the regulatory role and relevant mechanisms of LPA on GAG chain modification. We observed that in VSMCs, LPA

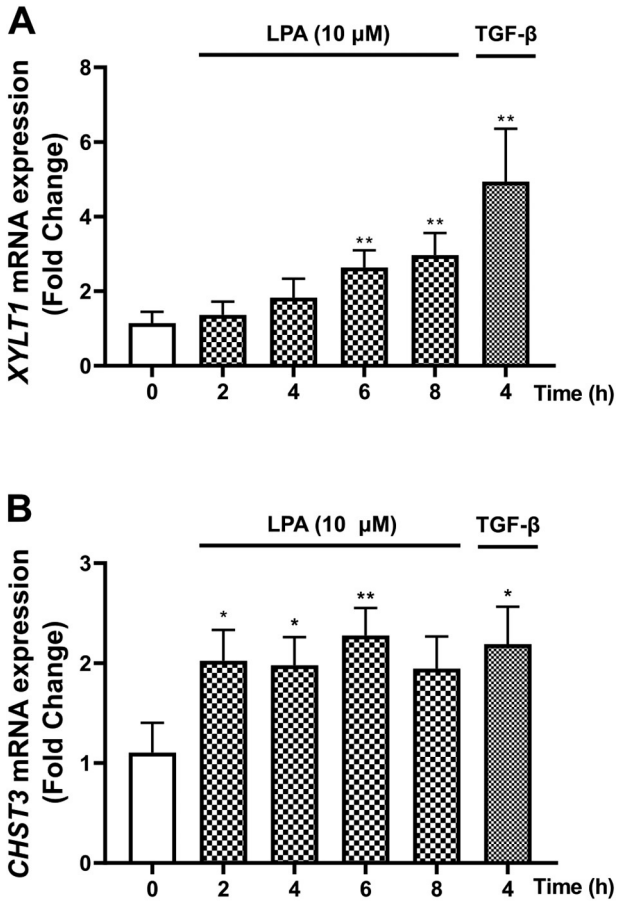

Fig. 5. LPA stimulates the mRNA expression of GAG chain synthesizing genes. RNA isolated from treated VMSCs was harvested and the mRNA expression of (A) *XYLT* and (B) *CHST3* was assessed using qRT-PCR. *18S* was used as the house keeping gene. Results are presented as mean ± SEM from four independent experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. *, $p < 0.05$ and **, $p < 0.01$ versus basal.

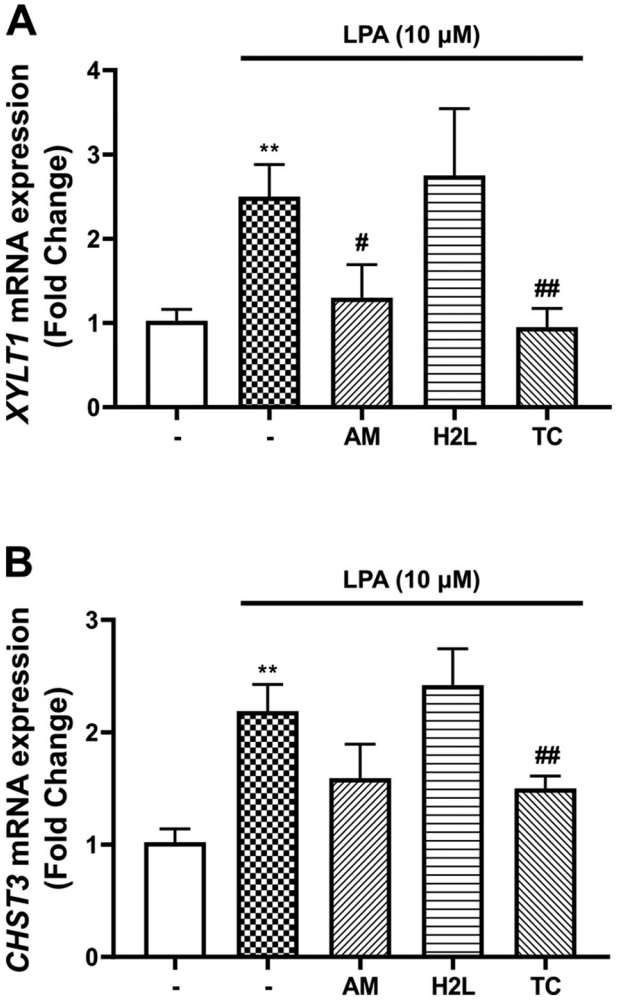

Fig. 6. The LPAR(s) involved in regulating the mRNA expression of GAG chain synthesizing genes. VSMCs cells were pre-incubated with AM095 (10 μM) (LPAR1 inhibitor), H2L5186303 (10 μM) (LPAR2 inhibitor) or TC LPA5 4 (10 μM) (LPAR5 inhibitor) for 30 min before exposure to LPA (10 μM) for 6 h. Total RNA was harvested and mRNA expression of (A) *XYLT* and (B) *CHST3* was assessed by qRT-PCR analysis. Results are expressed as mean ± SEM from four independent experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. **, $p < 0.01$ versus basal; #, $p < 0.05$ and ##, $p < 0.01$ versus LPA treated sample.

rapidly transactivates the TGFBR1 but not the EGFR. We have demonstrated that although human VSMCs express LPAR1, LPAR2 and LPAR5, transactivation of the TGFBR1 is occurring via LPAR5. LPAR5 mediated transactivation of the TGFBR1 leads to the subsequent formation of carboxyl-terminal phosphorylated Smad2 via ROCK dependent signalling pathways. LPAR5 mediated TGFBR1 activation upregulates the mRNA expression of genes associated with GAG chain initiation and elongation (Fig. 8).

The significance of GPCR transactivation dependent signalling is on the rise. Since the original observations of GPCR transactivation of the PTKR, this signalling paradigm has been expanded to include S/TKR transactivation [4,48]. Our recent work with GPCR agonist thrombin [49] demonstrated that transactivation dependent signalling contributes to 50% of gene expression and GPCR transactivation of PTKR was equally as important as transactivation of S/TKR with approximately 50% of the signalling occurring via each of the receptor pathways [49]. We have demonstrated in VSMCs, thrombin via its receptor PAR-1 transactivates the EGFR and TGFBR1 to stimulate GAG synthesizing gene expression [12,42] and GAG chain elongation [10,28]. LPA transactivates the TGFBR1 in mouse proximal tubule cells [50], human bronchial epithelial cells [30] and human airway smooth muscle cells [31]. Our data demonstrates that LPA specifically via LPAR5 transactivates the TGFBR1 to stimulate the phosphorylation of Smad2 carboxyl terminal. In our VSMC model, LPA stimulated pErk1/2 was not inhibited by EGFR antagonist, AG1478, demonstrating that LPA does not transactivate the EGFR. Several studies have demonstrated that LPA transactivates other PTKRs to stimulate pErk1/2 [51–54]. In mesangial, bronchial epithelial and smooth muscle cells, LPA transactivates PDGFR to stimulate pErk1/2 [52,53,55] and in CHO-K1 cells, LPA transactivates insulin-like growth factor-I receptor to stimulate downstream Erk1/2 signalling [51]. Belonging to GPCRs, LPARs can activate Erk1/2 dependent signalling pathways in the absence of transactivation dependent signalling [54,56]. In adipocytes, LPA mediated pErk1/2 was independent of EGFR or PDGFR transactivation; however, it was dependent on the activation of downstream G proteins [54]. These studies identify potential sources of LPA mediated pErk1/2 in VSMCs.

Several biochemical mechanisms are involved in the activation of the TGFBR1 [41]. The activation of Akt and its downstream associated effector AS160 drive cell surface translocation of TGFBRs. TGF-β [35], glucose [57] and insulin [36] drive intracellular TGFBRs to the cell surface increasing the sensitivity to autocrine or exogenous TGF-β. LPA is a potent stimulator of Akt, however we observed that LPA mediated transactivation of the TGFBR1 was not dependent on Akt pathways. This data shows that LPA mediated phosphorylation of Akt is not driving intracellular TGFBR1 to the cell surface to enhance TGF-β

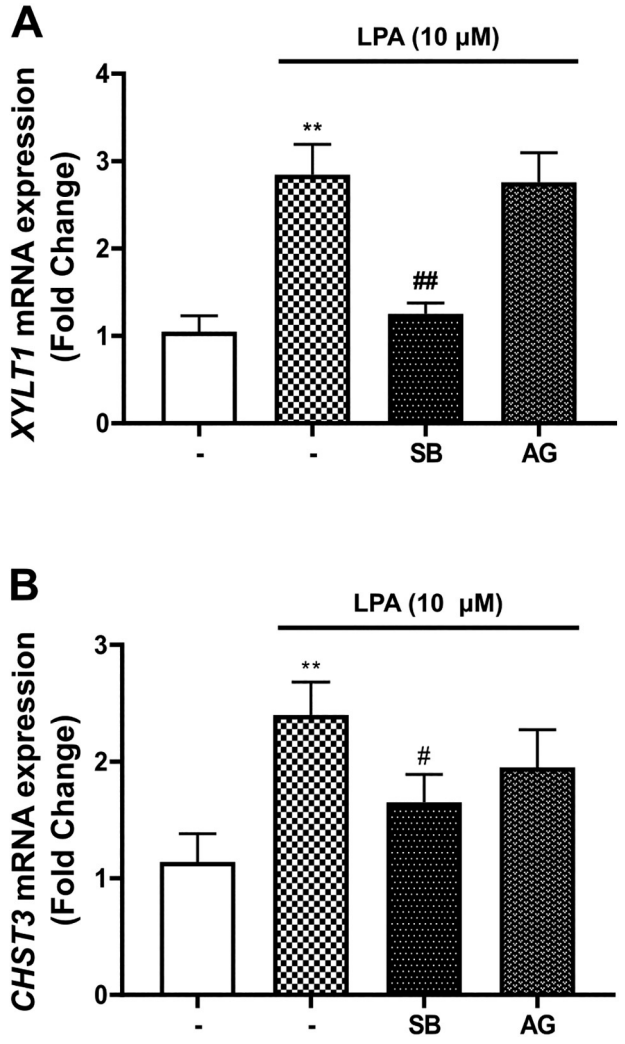

Fig. 7. The involvement of TGFBR1 transactivation pathway in GAG chain synthesizing gene expression. VSMCs cells were pre-incubated with SB431542 (3 μM) or AG1478 (5 μM) for 30 min before exposure to LPA (10 μM) for 6 h. Total RNA was harvested and the mRNA expression of (A) *XYLT1* and (B) *CHST3* was analysed using qRT-PCR. *18S* was used as a house keeping gene. Results are expressed as mean ± SEM from four independent experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. **, $p < 0.01$ versus basal; #, $p < 0.05$ and ##, $p < 0.01$ versus LPA treated sample.

responsiveness and the phosphorylation of Smad2. Activation of the TGFBR1 occurs via proteolytic cleavage of the latent TGF-β by MMPs [41]; however, us and others [12,28,42] have shown that GPCR transactivation of the TGFBR1 occurs independently of MMPs. An increase in ROS production promotes TGF-β signalling by oxidation of the large latent complex and release of TGF-β [40,41,58]. We demonstrate that LPA stimulates ROS production, whereas we observed that NOX/ROS dependent pathways are not involved in LPAR transactivation of the TGFBR1. In VSMCs, thrombin mediated TGFBR1 transactivation is regulated by Rho/ROCK signalling and downstream cytoskeletal mediated integrin activation [28,35]. The activated integrin binds to the RGD domain of the latent TGF-β complex to release TGF-β which then evokes TGFBR1 to elicit downstream responses [59]. Our data demonstrates the LPAR transactivation of the TGFBR1 is dependent on ROCK signalling pathways. This data highlights similarities in the biochemical mechanisms associated in thrombin and LPA transactivation of the TGFBR1.

LPA receptors LPAR1, LPAR2 and LPAR5 are the most abundantly expressed receptors in human VSMCs. However, when measuring the direct downstream response of the respective GPCR, intracellular calcium, we observed that only LPAR1 and LPAR5 are biologically functional in human VSMCs. LPAR transactivation dependent signalling is highly specific and we observed that LPAR5 but not LPAR1 can transactivate the TGFBR1. In mouse proximal tubule cells [50] and in human bronchial epithelial cells [30], LPA via LPAR2 induces ROCK/αVβ6 integrin mediated TGFBR1 transactivation. In human airway smooth muscle cells, LPA induces TGFBR1 transactivation via αVβ5 integrin [31]. These findings strengthen the status of a common biochemical mechanism in all GPCR transactivation of the TGFBR1 involving ROCK/integrin activation. As such, these signalling effectors represent potential therapeutic targets to inhibit pathophysiological effects of GPCR transactivation of the TGFBR1.

GAG chain elongation and the mRNA expression of GAG chain synthesizing genes correlate with atherosclerosis severity in vivo [20,60]. Traditional cardiovascular agonists such as thrombin [42,61], PDGF [25] and TGF-β [27,62] stimulate GAG chain elongation, LPA has a prominent role in promoting atherosclerosis and cardiovascular disease [2]. Our data demonstrates that in VSMCs, LPA stimulates the initiation and elongation of GAG chains on proteoglycans evidenced by an increase in the mRNA expression of *XYLT1* and *CHST3*. LPA mediated transactivation of the TGFBR1 occurred predominantly via the LPAR5; however, LPA mediated GAG gene expression was partially regulated by both LPAR1 and LPAR5. This demonstrates that LPAR1 via transactivation independent pathway can stimulate the expression of GAG chain synthesizing enzymes. The data together demonstrates that LPA via transactivation dependent and independent pathways drives the modification and elongation of GAG chains providing a novel perspective of the pathogenic role of LPA in the development of atherosclerosis.

5. Conclusions

In VSMCs, LPA via LPAR5 leads to the transactivation of the TGFBR1. We demonstrated that LPA via transactivation dependent and independent pathways stimulates the mRNA expression of rate limiting enzymes associated in GAG chain modification highlighting the contribution of LPA to early pathogenesis of atherosclerosis. We show that GPCR transactivation of the TGFBR1 occurs via a common biochemical mechanism involving ROCK/integrin signalling. The identified signalling effectors represent potential therapeutic targets to inhibit pathophysiological effects of GPCR transactivation of the TGFBR1.

CRediT authorship contribution statement

Ying Zhou: Data curation, Visualization, Writing - original draft. **Peter J. Little:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Yingnan Cao:** Conceptualization, Writing - review & editing. **Hang T. Ta:** Supervision, Writing - review & editing. **Danielle Kamato:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Funding supporting the studies in our laboratory and referenced in this review has been received from the National Health and Medical Research Council (1022800) of Australia, National Heart Foundation of

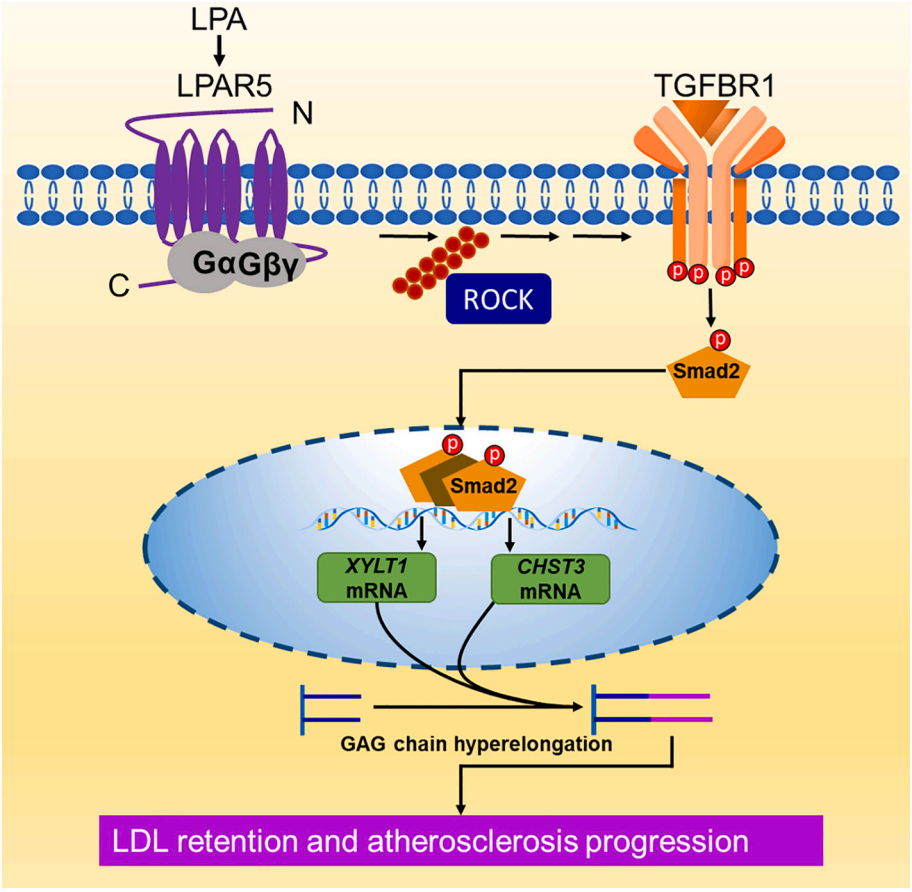

Fig. 8. A schematic of LPAR mediated transactivation of the TGFBR1 stimulates the mRNA expression of genes that are critical in the development of atherosclerosis. In VSMCs, LPAR5 transactivates the TGFBR1 via ROCK dependent pathways. LPAR5 transactivation of the TGFBR1 leads to the phosphorylation of Smad2C and a stimulation in atherosclerosis associated GAG chain initiation gene *XYLT1* and elongation gene *CHST3*.

Australia (G09M4385), Diabetes Australia Research Trust (PJL), Academic support packages the University of Queensland (PJL). YZ is supported by the Research Training Scholarship of The University of Queensland. DK is supported by NHMRC-Peter Doherty (1160925) and National Heart Foundation of Australia (102129) fellowships. HTT is funded by National Heart Foundation of Australia (102761).

References

[1] W.H. Moolenaar, Lysophosphatidic acid, a multifunctional phospholipid messenger, J. Biol. Chem. 270 (1995) 12949–12953.
[2] Y. Zhou, et al., Lysophosphatidic acid and its receptors: pharmacology and therapeutic potential in atherosclerosis and vascular disease, Pharmacol. Ther. 204 (2019) Epub ahead of print.
[3] J.W. Choi, et al., LPA receptors: subtypes and biological actions, Annu. Rev. Pharmacol. Toxicol. 50 (2010) 157–186.
[4] D. Kamato, et al., The expansion of GPCR transactivation-dependent signalling to include serine/threonine kinase receptors represents a new cell signalling frontier, Cell. Mol. Life Sci. 72 (2015) 799–808.
[5] A.G. Gilman, G proteins: transducers of receptor-generated signals, Annu. Rev. Biochem. 56 (1987) 615–649.
[6] R.J. Lefkowitz, S.K. Shenoy, Transduction of Receptor Signals by ß-Arrestins, Science, 2005.
[7] H. Daub, et al., Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors, Nature 379 (1996) 557–560.
[8] P.J. Little, et al., The paradigm of g protein receptor transactivation: a mechanistic definition and novel example, ScientificWorldJournal 11 (2011) 709–714.
[9] P.J. Little, et al., Integrating the GPCR transactivation-dependent and biased signalling paradigms in the context of PAR-1 signalling, Br. J. Pharmacol. 173 (2015) 2992–3000.
[10] M.L. Burch, et al., Thrombin stimulation of proteoglycan synthesis in vascular smooth muscle is mediated by PAR-1 transactivation of the transforming growth factor βtype I receptor, J. Biol. Chem. 285 (2010) 26798–26805.
[11] P.J. Little, et al., Endothelin-1 stimulation of proteoglycan synthesis in vascular smooth muscle is mediated by endothelin receptor transactivation of the transforming growth factor-β type I receptor, J. Cardiovasc. Pharmacol. 56 (2010) 360–368.
[12] D. Kamato, et al., Protease activated receptor-1 mediated dual kinase receptor transactivation stimulates the expression of glycosaminoglycan synthesizing genes, Cell. Signal. 28 (2016) 110–119.
[13] R. Wetzker, F.-D. Böhmer, Transactivation joins multiple tracks to the ERK/MAPK cascade, Nat. Rev. Mol. Cell Biol. 4 (2003) 651–657.
[14] S.J. Forrester, et al., Epidermal growth factor receptor transactivation: mechanisms, pathophysiology, and potential therapies in the cardiovascular system, Annu. Rev. Pharmacol. Toxicol. 56 (2016) 627–653.
[15] J. Borén, K.J. Williams, The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: a triumph of simplicity, Curr. Opin. Lipidol. 27 (2016) 473–483.
[16] P.J. Little, N. Osman, K.D. O'Brien, Hyperelongated biglycan: the surreptitious initiator of atherosclerosis, Curr. Opin. Lipidol. 19 (2008) 448–454.
[17] K.J. Williams, I. Tabas, The response-to-retention hypothesis of early atherogenesis, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 551–561.
[18] S.N. Yang, et al., Growth factor-mediated hyper-elongation of glycosaminoglycan chains on biglycan requires transcription and translation, Arch. Physiol. Biochem. 115 (2009) 147–154.
[19] R. Getachew, et al., PDGF β-receptor kinase activity and ERK1/2 mediate glycosaminoglycan elongation on biglycan and increases binding to LDL, Endocrinology 151 (2010) 4356–4367.
[20] M.L. Ballinger, et al., Imatinib inhibits vascular smooth muscle proteoglycan synthesis and reduces LDL binding in vitro and aortic lipid deposition in vivo, J. Cell. Mol. Med. 14 (2010) 1408–1418.
[21] R. Afroz, et al., Signalling pathways regulating galactosaminoglycan synthesis and structure in vascular smooth muscle: implications for lipoprotein binding and atherosclerosis, Pharmacol. Ther. 187 (2018) 88–97.
[22] T. Mikami, H. Kitagawa, Biosynthesis and function of chondroitin sulfate, Biochim. Biophys. Acta 1830 (2013) 4719–4733.
[23] H. Kitagawa, et al., Sulfation of the galactose residues in the glycosaminoglycan-protein linkage region by recombinant human chondroitin 6-O-sulfotransferase-1, J. Biol. Chem. 283 (2008) 27438–27443.
[24] M.E. Ivey, P.J. Little, Thrombin regulates vascular smooth muscle cell proteoglycan synthesis via PAR-1 and multiple downstream signalling pathways, Thromb. Res. 123 (2008) 288–297.
[25] P.J. Little, et al., Suramin inhibits PDGF-stimulated receptor phosphorylation, proteoglycan synthesis and glycosaminoglycan hyperelongation in human vascular smooth muscle cells, J. Pharm. Pharmacol. 65 (2013) 1055–1063.
[26] M.L. Burch, et al., TGF-β stimulates biglycan synthesis via p38 and ERK phosphorylation of the linker region of Smad2, Cell. Mol. Life Sci. 67 (2010) 2077–2090.
[27] M.A. Rostam, et al., Flavopiridol inhibits TGF-β-stimulated biglycan synthesis by blocking linker region phosphorylation and nuclear translocation of Smad2, J. Pharmacol. Exp. Ther. 365 (2018) 156–164.

[28] M.L. Burch, et al., Thrombin-mediated proteoglycan synthesis utilizes both protein-tyrosine kinase and serine/threonine kinase receptor transactivation in vascular smooth muscle cells, J. Biol. Chem. 288 (2013) 7410–7419.
[29] R. Mohamed, et al., ROS directly activates transforming growth factor beta type 1 receptor signalling in human vascular smooth muscle cells, Biochim. Biophys. Acta Gen. Subj. 1864 (2020) Epub ahead of print.
[30] M.Y. Xu, et al., Lysophosphatidic acid induces αvβ6 integrin-mediated TGF-β activation via the LPA2 receptor and the small G protein Gαq, Am. J. Pathol. 174 (2009) 1264–1279.
[31] A.L. Tatler, et al., Integrin αvβ5-mediated TGF-β activation by airway smooth muscle cells in asthma, J. Immunol. 187 (2011) 6094–6107.
[32] G.J. Inman, et al., SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7, Mol. Pharmacol. 62 (2002) 65–74.
[33] C.-I. Lin, et al., Lysophosphatidic acid upregulates vascular endothelial growth factor-C and tube formation in human endothelial cells through LPA1/3, COX-2, and NF-κB activation-and EGFR transactivation-dependent mechanisms, Cell. Signal. 20 (2008) 1804–1814.
[34] N. Osherov, A. Levitzki, Epidermal-growth-factor-dependent activation of the Src-family kinases, Eur. J. Biochem. 225 (1994) 1047–1053.
[35] D. Duan, R. Derynck, Transforming growth factor-β (TGF-β)-induced up-regulation of TGF-β receptors at the cell surface amplifies the TGF-β response, J. Biol. Chem. 294 (2019) 8490–8504.
[36] E.H. Budi, B.P. Muthusamy, R. Derynck, The insulin response integrates increased TGF-β signaling through Akt-induced enhancement of cell surface delivery of TGF-β receptors, Sci. Signal. 8 (2015) ra96.
[37] H. Hirai, et al., MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo, Mol. Cancer Ther. 9 (2010) 1956–1967.
[38] K. Lin, et al., An ATP-site on-off switch that restricts phosphatase accessibility of Akt, Sci. Signal. 5 (2012) Ra37.
[39] N. Rhodes, et al., Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity, Cancer Res. 68 (2008) 2366–2374.
[40] I.B. Robertson, D.B. Rifkin, Regulation of the bioavailability of TGF-β and TGF-β-related proteins, Cold Spring Harb. Perspect. Biol. 8 (2016) a021907.
[41] J.P. Annes, J.S. Munger, D.B. Rifkin, Making sense of latent TGFbeta activation, J. Cell Sci. 116 (2003) 217–244.
[42] D. Kamato, et al., Mechanisms of PAR-1 mediated kinase receptor transactivation: Smad linker region phosphorylation, J. Cell Commun. Signal. 13 (2019) 539–548.
[43] R. Mohamed, et al., Transforming growth factor-beta1 mediated CHST11 and CHSY1 mRNA expression is ROS dependent in vascular smooth muscle cells, J. Cell Commun. Signal. 13 (2018) 225–233.
[44] C.C. Lin, et al., Lysophosphatidic acid induces reactive oxygen species generation by activating protein kinase C in PC-3 human prostate cancer cells, Biochem. Biophys. Res. Commun. 440 (2013) 564–569.
[45] Q. Chen, N. Olashaw, J. Wu, Participation of reactive oxygen species in the lysophosphatidic acid-stimulated mitogen-activated protein kinase kinase activation pathway, J. Biol. Chem. 270 (1995) 28499–28502.
[46] C.L. Chang, et al., Lysophosphatidic acid-induced interleukin-1 beta expression is mediated through Gi/Rho and the generation of reactive oxygen species in macrophages, J. Biomed. Sci. 15 (2008) 357–363.
[47] U. Kaneyuki, et al., Pitavastatin inhibits lysophosphatidic acid-induced proliferation and monocyte chemoattractant protein-1 expression in aortic smooth muscle cells by suppressing Rac-1-mediated reactive oxygen species generation, Vasc. Pharmacol. 46 (2007) 286–292.
[48] R. Chaplin, et al., Insights into cellular signalling by G protein coupled receptor transactivation of cell surface protein kinase receptors, J. Cell Commun. Signal. 11 (2017) 117–125.
[49] D. Kamato, et al., RNA sequencing to determine the contribution of kinase receptor transactivation to G protein coupled receptor signalling in vascular smooth muscle cells, PLoS One 12 (2017) e0180842.
[50] H. Geng, et al., Lysophosphatidic acid increases proximal tubule cell secretion of profibrotic cytokines PDGF-B and CTGF through LPA2-and Gαq-mediated Rho and αvβ6 integrin-dependent activation of TGF-β, Am. J. Pathol. 181 (2012) 1236–1249.
[51] M.C. Olianas, S. Dedoni, P. Onali, Antidepressants activate the lysophosphatidic acid receptor LPA1 to induce insulin-like growth factor-I receptor transactivation, stimulation of ERK1/2 signaling and cell proliferation in CHO-K1 fibroblasts, Biochem. Pharmacol. 95 (2015) 311–323.
[52] M. Goppelt-Struebe, S. Fickel, C.O. Reiser, The platelet-derived-growth-factor receptor, not the epidermal-growth-factor receptor, is used by lysophosphatidic acid to activate p42/44 mitogen-activated protein kinase and to induce prostaglandin G/H synthase-2 in mesangial cells, Biochem. J. 345 (2000) 217–224.
[53] L. Wang, et al., Involvement of phospholipase D2 in lysophosphatidate-induced transactivation of platelet-derived growth factor receptor-beta in human bronchial epithelial cells, J. Biol. Chem. 278 (2003) 39931–39940.
[54] T.E. Holmström, et al., Non-transactivational, dual pathways for LPA-induced Erk1/2 activation in primary cultures of brown pre-adipocytes, Exp. Cell Res. 316 (2010) 2664–2675.
[55] F. Hao, et al., LPA induces IL-6 secretion from aortic smooth muscle cells via an LPA1-regulated, PKC-dependent, and p38alpha-mediated pathway, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) 974–983.
[56] Z.G. Goldsmith, D.N. Dhanasekaran, G protein regulation of MAPK networks, Oncogene 26 (2007) 3122–3142.
[57] L. Wu, R. Derynck, Essential role of TGF-beta signaling in glucose-induced cell hypertrophy, Dev. Cell 17 (2009) 35–48.
[58] R.-M. Liu, L.P. Desai, Reciprocal regulation of TGF-β and reactive oxygen species: a perverse cycle for fibrosis, Redox Biol. 6 (2015) 565–577.
[59] M. Shi, et al., Latent TGF-β structure and activation, Nature 474 (2011) 343–349.
[60] V.Y. Anggraeni, et al., Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis, Biochem. Biophys. Res. Commun. 406 (2011) 36–41.
[61] D. Kamato, et al., Individual Smad2 linker region phosphorylation sites determine the expression of proteoglycan and glycosaminoglycan synthesizing genes, Cell. Signal. 53 (2019) 365–373.
[62] M.A. Rostam, et al., The role of specific Smad linker region phosphorylation in TGF-β mediated expression of glycosaminoglycan synthesizing enzymes in vascular smooth muscle, Cell. Signal. 28 (2016) 956–966.