BBA - General Subjects 1864 (2020) 129463

Contents lists available at ScienceDirect

BBA - General Subjects

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

ROS directly activates transforming growth factor β type 1 receptor signalling in human vascular smooth muscle cells

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ARTICLE INFO

Keywords:
Apocynin
NADPH oxidase
Reactive oxygen species
Smad2 carboxy terminal
Smooth muscle cells
Transforming growth factor β type 1 receptor

ABSTRACT

Background: Widely used NAPDH oxidase (Nox) inhibitor, apocynin is a prodrug that needs to be converted to its pharmacologically active form by myeloperoxidase. In myeloperoxidase deficient non phagocytic cells such as vascular smooth muscle cells (VSMCs) apocynin stimulates the production of ROS. ROS is generated by the activation of many signalling pathways, thus we have used apocynin as a pharmacological tool to characterise the role of endogenous ROS in activating the transforming growth factor beta receptor (TGFBR1) without the activation of other pathways.

Methods: The in vitro study utilized human VSMCs. Western blotting and quantitative real time PCR were performed to assess signalling pathways and gene expression, respectively. Intracellular ROS levels was measured using fluorescence detection assay.

Results: Treatment with apocynin of human VSMCs stimulated ROS production and the phosphorylation of TGFBR1 and subsequent activation of TGFBR1 signalling leading to the formation of phosphorylated Smad2 which consequently upregulates the mRNA expression of glycosaminoglycan synthesizing enzyme.

Conclusions: These findings outline a specific involvement of ROS production in TGFBR1 activation. Furthermore, because apocynin stimulates Nox and ROS production, apocynin must be used with considerable care in vitro as its actions clearly extend beyond the stimulation of Nox enzymes and it has consequences for cellular signalling.

General significance: Apocynin can stimulate Nox leading to the production of ROS and the outcome is completely dependent upon the redox properties of the cell.

1. Introduction

G protein coupled receptors (GPCRs) are the most prolific receptor families in biology [1,2] and they are the targets for largest number of therapeutic agents reflecting their role in multiple pathophysiologies [3]. The original paradigm of classic GPCR signalling [4] has been enormously broadened with the acknowledgment of GPCR transactivation dependent signalling [5]. The paradigm of GPCR transactivation signalling was originally established in the context of protein tyrosine kinase receptors (PTKR) stimulation notably epidermal growth factor receptor (EGFR) [5–7] and has since and recently been extended to include the transactivation of S/TKR, specifically transforming growth factor beta type one receptor (TGFBR1) [8–10]. This transactivation mechanism further extends the responses attributable to

Abbreviations: CHST11, Chondroitin-4-sulfotransferase-1; DPI, diphenyleneiodonium; EGFR, epidermal growth factor receptor; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; MPO, myeloperoxidase; NAC, *N*-acetyl-L-cysteine; Nox, nicotinamide adenine dinucleotide phosphate oxidase; PTKR, protein tyrosine kinase receptor; ROS, reactive oxygen species; S/TKR, serine/threonine kinase receptor; TGFBR1, transforming growth factor-β type 1 receptor; TGF-β, transforming growth factor β; VSMCs, vascular smooth muscle cells; XYLT1, Xylotransferase-1

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https://doi.org/10.1016/j.bbagen.2019.129463
Received 11 July 2019; Received in revised form 25 September 2019; Accepted 7 October 2019
Available online 30 October 2019
0304-4165/

GPCRs to include fibrosis, atherogenesis and cancer metastases [11,12]. Surprisingly, mechanistic studies have thus far revealed that the biochemical processes of these two GPCR to kinase receptor transactivation pathways are completely distinct [13–16]. It will be necessary to fully characterise and understand these mechanisms of transactivation signalling in order to reveal a potential therapeutic target for all of the diseases in which these transactivation processes are involved. Reactive oxygen species (ROS) are highly reactive molecules that act as second messengers to modulate signalling pathways. ROS are involved in GPCR transactivation of PTKR, however the role of ROS in GPCR transactivation of the TGFBR1 has not yet been explored.

ROS are generated by the action of many growth factors and hormones but studying the role of ROS under these circumstances is heavily confounded by the many pathways that are concomitantly activated by the relevant growth factor or hormone. We wished to explore the role of Nox and ROS in transforming growth factor β (TGF-β) signalling in a cellular environment not confounded by the concurrent activation of multiple other activated pathways as would occur in cells treated with growth factors to increase ROS. Accordingly, we explored an earlier observation [17] and examined the role of apocynin in stimulating Nox and generating ROS in human vascular smooth muscle cells (VSMCs).

Apocynin (4-hydroxy-3-methoxyacetophenone) is a catechol natural product which can be extracted from the roots of *Picrorrhiza kurroa* [18]. Apocynin is very widely used in vitro and in vivo [19,20] as an inhibitor of NADPH Oxidase (Nox). However, in spite of its wide use as a Nox inhibitor, convincing data demonstrates that Apocynin stimulates the production of ROS in non-phagocytic cells [17]. Apocynin stimulated ROS production in vascular fibroblasts which was inhibited by NADPH inhibitor diphenyleneiodonium (DPI), indicates that the apocynin stimulated ROS was derived from Nox [17]. These studies have been extended to identify that non-phagocytic vascular cells lack myeloperoxidase which is required for the dimerization of apocynin [21]. Taken together these studies identify a role for apocynin as a stimulator of ROS in non-phagocytic vascular cells and this property will be used to study the independent role of ROS/Nox activation of the TGFBR1 in VSMCs.

Our earlier work characterising the mechanism of GPCR activation of the TGFBR1 demonstrated that GPCRs activate cytoskeletal rearrangement which activates ROCK signalling leading to the activation of cell surface integrins which bind to the large latent TGF-β complex [16,22]. In VSMCs this transactivation pathway leads to the phosphorylation of transcription factor Smad2 in the carboxy terminus and the expression of genes associated with the elongation of glycosaminoglycan (GAG) chains on proteoglycans such as biglycan [23]. Here we confirmed in detail the earlier studies in relation to apocynin stimulation of ROS in VSMCs, and then explored the role of ROS in the activation of TGFBR1 signalling. These experiments are conducted in the context of the role of TGF-β and Smad2 carboxyl-terminal phosphorylation [24–27] in stimulating the hyperelongation of GAG chains on the proteoglycan biglycan through the expression of the rate limiting enzyme chondroitin-4-sulfotransferase-1 (CHST11). Hyperelongated GAG chains on biglycan lead to enhanced binding of low density lipoproteins (LDL) such that this is an in vitro model of atherosclerosis [28–32]. In this study, we confirmed and extended the observation that apocynin stimulates Nox leading to an increase in ROS levels; this leads to an activation of TGF-β signalling producing *bone fide* cellular responses such as GAG chain elongation.

2. Materials and methods

2.1. Materials

Ham's F-12 K (Kaighn's) medium, antibiotics solution (10,000 U/ml penicillin, 10,000 μg/ml streptomycin), 0.25% trypsin-EDTA (1 ×) and fetal bovine serum (FBS), Pierce™ bicinchoninic acid protein assay kit were purchased from ThermoFisher Scientific (VIC, AUS). The following chemicals were obtained from Sigma Aldrich (NSW, AUS): SB431542, apocynin, DPI, ML-171, N-Acetyl-Cysteine (NAC), 2`,7`-Dichlorofluorescence diacetate (H_2DCFDA) dye, HEPES ≥99.5% (titration), sodium chloride (NaCl), sodium hydroxide, calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), D-(+)-glucose, sodium dodecyl sulfate (SDS), 2-mercaptoethanol and dimethyl sulphoxide. Potassium chloride (KCl) was from Chem-supply Pty Ltd. (SA, AUS). Human recombinant transforming growth factor-β, anti-phospho-Smad2 (Ser465/467), anti-phospho-Ezrin(Thr567)/Radixin(Thr567)/Moesin (Thr558), anti-rabbit-immunoglobulin G horseradish peroxidase and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal IgG antibody were purchased from Australian Biosearch (WA, AUS). The BioRad Trans-Blot® Turbo RTA transfer kit, polyvinylidene fluoride (PVDF) membrane, 30% acrylamide/bis-acrylamide solution, N,N,N`,N`-tetramethylehtylenediamine (TEMED), Ammonium persulfate (APS) and Quantity one imaging software were from BioRad laboratories (VIC, AUS). Primers for housekeeping gene 18S and CHST11 gene, Quantitect® reverse transcription kit and QuantiNova™ Sybr® green PCR (2500) kit were purchased from Qiagen (VIC, AUS).

2.2. Culture of human aortic vascular smooth muscle cells

Human aortic-vascular smooth muscle cells (HA-VSMCs) (ATCC®-CRL 1999™) were purchased from In Vitro Technologies Life science (VIC, AUS). These cells were grown in F-12 K culture medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in 5% CO_2. VSMCs were passaged to provide sufficient numbers for experimentation. VSMCs were seeded in 60 mm dishes or onto 96 well plate and maintained until confluent. Confluent cultures were serum starved in Ham's F-12 K medium containing 0.1% FBS and 1% penicillin-streptomycin for 48 h before treatment.

2.3. Western blotting

Total cell lysates with concentration of 20 μg were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. Non-specific binding sites were blocked with 5% BSA for 1 h at room temperature and primary antibodies were incubated overnight at 4 °C followed by peroxidase labelled anti-rabbit IgG and enhanced Chemiluminescence (ECL) detection. The membranes were then reprobed with anti-GAPDH. Bio-Rad gel documentation was used for imaging the blots and densitometry examination was performed using Image Lab imaging software.

2.4. Assessing mRNA gene expression

The mRNA level of CHST11 was determined by quantitative real-time polymerase chain reaction (q-RT-PCR). To measure gene expression, total RNA from cultured cells was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. RNA concentration and purity were checked using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). First strand cDNA was synthesized from 500 ng RNA using the Quantitect® reverse transcription kit (Qiagen) according to the manufacturer's protocol. qRT-PCR was performed using Qiagen Rotor gene Q and QuantiNova™ Sybr® green PCR kit (Qiagen) together with specific primers. Data was normalized to the 18S as the housekeeping gene. All experiments were performed at least four times and analysis performed in duplicate for each experiment. The delta-delta cycle-threshold (ΔΔCt) method was used to analyse the fold change in mRNA expression from qRT-PCR experiments.

2.5. Intracellular ROS detection assay

Quiescent VSMCs were washed with 100 μl of Krebs-HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$,

11 mM D-(+)-glucose and pH 7.4) and incubated for 45 min with 10 μM H2DCF-DA at 37 °C and 5% CO2. The dye was gently removed and replaced with Krebs-HEPES buffer in the presence and absence of respective. The fluorescence was measured intensity using Ensight Multimode Plate Reader at excitation/emission = 485/535 nm. The fluorescence intensity was used to calculate the fold change.

2.6. Statistical analysis

Data was normalized and shown as the mean ± standard error of the mean (SEM) of four independent experiments performed, unless stated otherwise. A One-Way Analysis of Variance was used to calculate statistical significance of normalized data as stated, followed by least significant difference post-hoc analysis. Results considered significant when the probability was < 0.05 ($^{*}p < 0.05$) and 0.01 ($^{**}p < 0.01$).

3. Results

3.1. Apocynin stimulates intracellular ROS production in human VSMCs

Apocynin has been widely utilized in experimental research as a Nox inhibitor [18]. However, in non-phagocyte cells, apocynin stimulates ROS production [17]. Here, we investigated the role of apocynin as a modulator of intracellular ROS production in human VSMCs. To address this question, VSMCs were treated with apocynin (10 μM) over a 4 h period (0–240 min) (Fig. 1A). Apocynin treated VSMCs rapidly increase ROS levels with a 1.5-fold ($p < 0.01$) increase observed at 15 min. Treatment with apocynin gradually increases ROS level with a peak of 2-fold ($p < 0.01$) observed at 60 min (Fig. 1A) after which it gradually declined to 1.5-fold ($p < 0.01$) at 240 min. Next VSMCs were treated with apocynin (1–100 μM) for 1 h (Fig. 1B). Treatment with apocynin dose dependently increased intracellular ROS levels in VSMCs with a 1.5-fold change ($p < 0.01$) observed with 3 μM treatment with an increase of up to 4.1-fold ($p < 0.01$) when treated with 100 μM apocynin (Fig. 1B). This data indicates that treatment of VSMCs with apocynin stimulates ROS production in a time and concentration dependent manner.

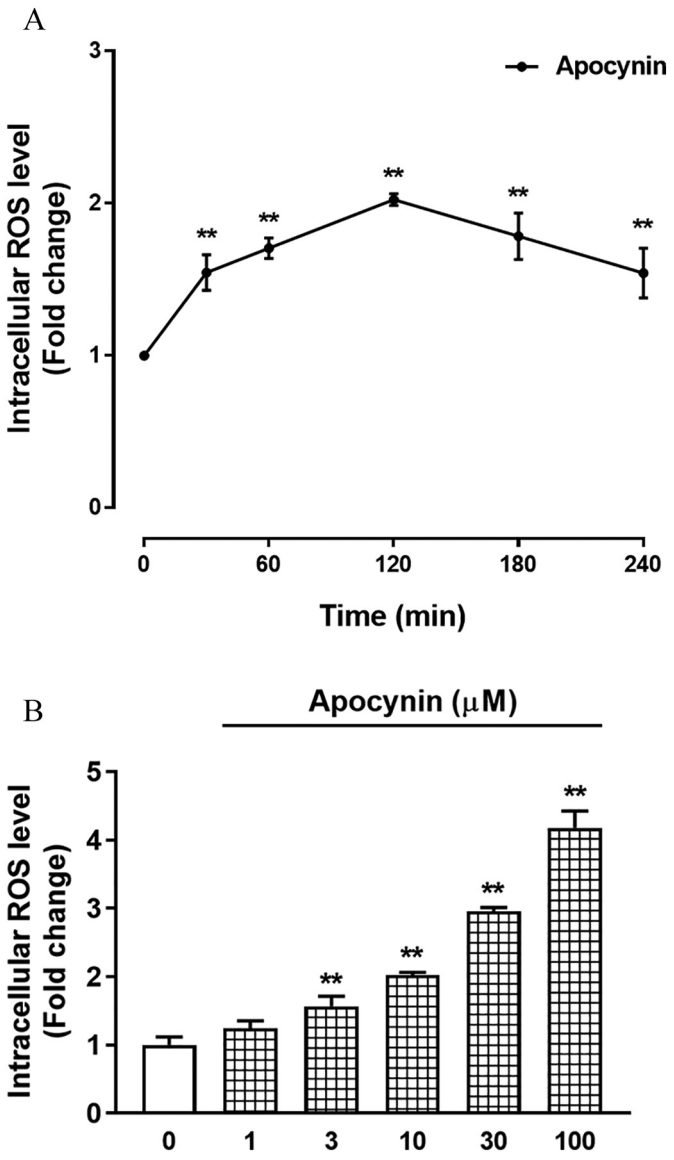

Fig. 1. Apocynin stimulates ROS generation in a time and concentration dependent manner in human vascular smooth muscle cells. (A) VSMCs were treated with apocynin (10 μM) for 15–240 min. (B) VSMCs were treated with apocynin (1–100 μM) for 1 h. Histogram represents fluorescence intensity minus the baseline, expressed as fold per basal level. Results are expressed as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA followed by least significant difference post-hoc analysis. $^{**}p < 0.01$ compared with untreated control.

3.2. Apocynin stimulates a Nox1-dependent increase in ROS generation in human VSMCs

The main source of ROS production in VSMCs are Nox enzymes [33]. Human VSMCs typically express Nox1, Nox2, Nox4 and Nox5 enzymes [34]. To investigate whether or not apocynin mediated ROS production in VSMCs was generated from Nox, pharmacological Nox inhibitors were utilized. VSMCs were treated in the presence and absence of the selective Nox1 antagonist, ML-171 (10 μM) [35], the broad Nox inhibitor, DPI (10 μM) [33] and the ROS scavenger, NAC (5 mM) followed by apocynin (10 μM) for 60 min (Fig. 2). In these experiments treatment of VSMCs with apocynin increased ROS levels to 2.1-fold ($p < 0.01$) as compared to non-treated control. The increase in apocynin mediated ROS levels were completely inhibited ($p < 0.01$) in the presence of ML-171, DPI and NAC. This result shows that apocynin mediated ROS production in VSMCs is derived from Nox enzymes.

3.3. Apocynin time dependently stimulates phosphorylation of TGFBR1 in human VSMCs

In multiple in vitro models, ROS activate TGF-β mediated signalling pathways [36]. To investigate this response very specifically, we investigated whether or not endogenous ROS production can phosphorylate the TGFBR1 in our VSMCs model. Human VSMCs were treated apocynin (10 μM) over a 2 h time period (0–120 min) (Fig. 3). Treatment with apocynin increased the phosphorylation of the TGFBR1 to 1.4-fold ($p < 0.05$) at 120 min (Fig. 3). VSMCs treated with TGF-β stimulated TGFBR1 phosphorylation to 1.7-fold ($p < 0.01$). Apocynin mediated TGFBR1 phosphorylation represented approximately 80% of full TGF-β effect (Fig. 3). This data show that apocynin mediated ROS production activates the TGFBR1 in human VSMCs.

3.4. Apocynin and hydrogen peroxide stimulate phosphorylation of Smad2 carboxyl-terminal in human VSMCs

We observed above that apocynin treated VSMCs activate the TGFBR1 (Fig. 3). Nox derived ROS production modulates TGFBR1 signalling pathways leading to the phosphorylation of Smads [37]. Hence, we investigated the actions of endogenous (apocynin) generated and exogenous (hydrogen peroxide) sources of ROS on the Smad signalling pathway in VSMCs. Treatment of VSMCs with apocynin over a 4 h time course increased the phosphorylation of the Smad transcription factor in the carboxyl-terminal. Treatment with apocynin increased the

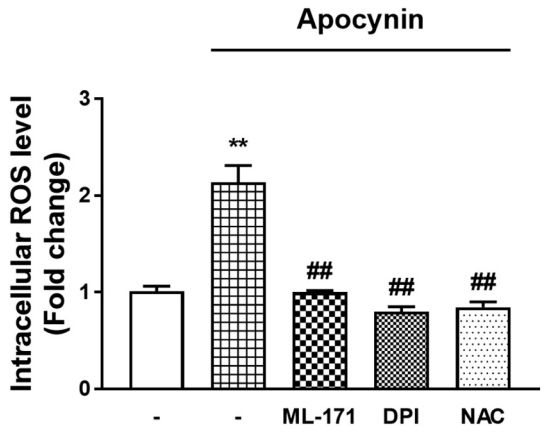

Fig. 2. Apocynin stimulates a Nox1 dependent increase in ROS generation in human VSMCs. VSMCs were treated with apocynin (10 μM) for 1 h in the presence and absence of the selective Nox1 inhibitor, ML-171 (10 μM), non-specific Nox inhibitor, DPI (10 μM) or ROS scavenger, NAC (5 mM). Histogram represents fluorescence intensity minus the baseline, expressed as fold per basal. Results are expressed as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA followed by least significant difference post-hoc analysis. $^{**}p < 0.01$ compared with untreated control and $^{\#\#}p < 0.01$ compared with apocynin.

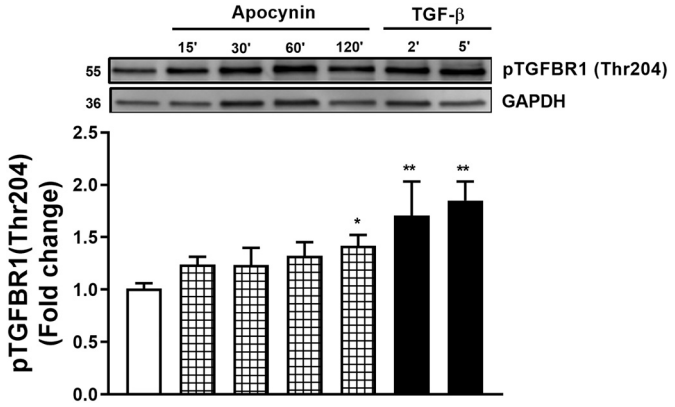

Fig. 3. Apocynin time-dependently stimulates phosphorylation of transforming growth factor receptor type 1 in human VSMCs. VSMCs were stimulated with apocynin (10 μM) for 0–120 min or TGF-β (2 ng/ml) for 2 and 5 min. Membranes were incubated with anti-phospho-TGFBR1 (Thr204) (1:500) and then followed by incubation with peroxidase labelled anti-rabbit IgG (1:2000) and ECL detection. Anti-GAPDH was used as a loading control. Normalized data in each case are shown as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA fooled by least significant difference post-hoc analysis. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with untreated control.

phosphorylation of Smad2 within 15 min ($p < 0.01$) (Fig. 4A) and continued to gradually increase until a peak stimulation of 2.5-fold ($p < 0.01$) was observed at 120 min post treatment. As expected TGF-β used as a control strongly stimulated Smad2 phosphorylation ($p < 0.01$). The 2 h time point was selected for all future pharmacological studies with apocynin. Treatment of VSMCs with apocynin at various concentrations (1–100 μM) increased the carboxy terminal phosphorylation of the Smad2 transcription factor (Fig. 4B). Treatment with apocynin at 1 μM stimulated Smad2 phosphorylation to 1.5-fold ($p < 0.01$) and a peak stimulation of 1.8-fold (p < 0.01) was observed with 10 μM treatment of apocynin (Fig. 4B). Tert-butyl hydrogen peroxide (TBHP) used to investigate the role of exogenous ROS on TGFBR1 pathways. VSMCs were treated with TBHP (100 μM) over a 2 h time course (Fig. 4C) and Smad2 carboxyl-terminal phosphorylation was assessed. Treatment of VSMCs with TBHP gradually increased Smad2

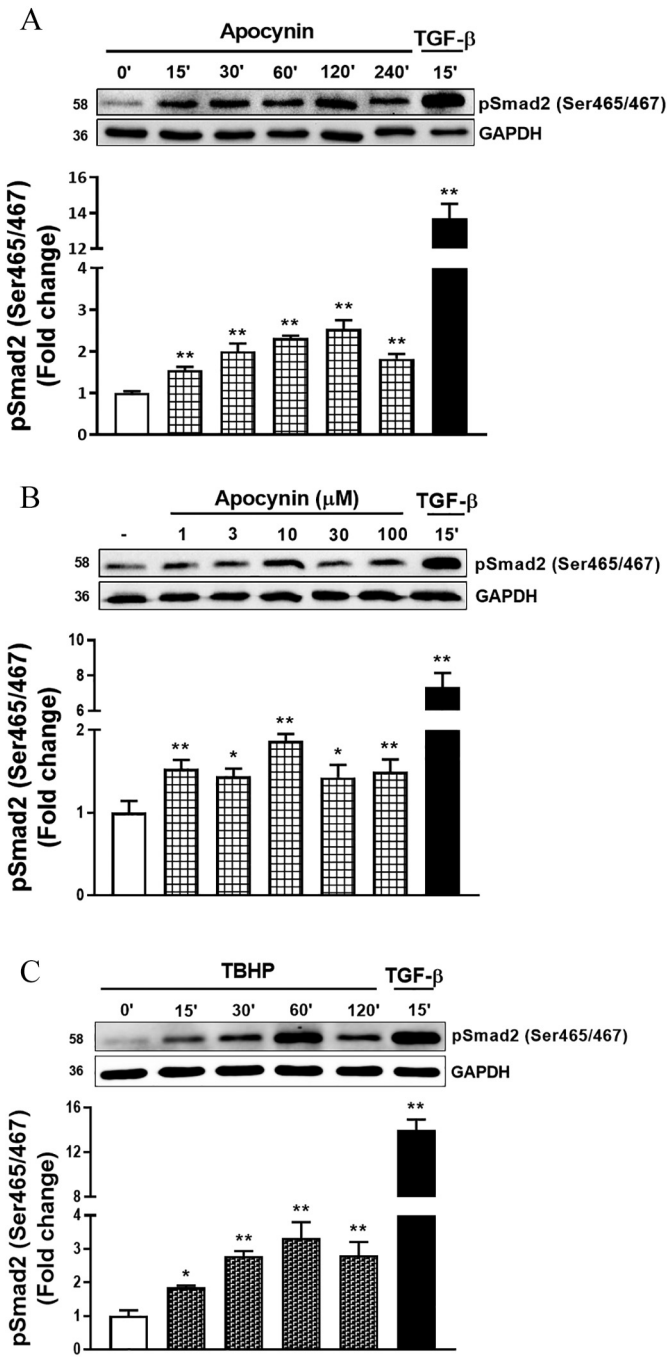

Fig. 4. Apocynin promotes carboxyl-terminal phosphorylation of Smad2 in a time- and concentration-dependent manner in human VSMCs. (A) VSMCs were stimulated with apocynin (10 μM) for 0–240 min or TGF-β (2 ng/ml) for 15 min. (B) VSMCs were stimulated with apocynin (1–100 μM) for 2 h or 2 ng/ml TGF-β for 15 min. (C) VSMCs were stimulated with TBHP (100 μM) for 0–120 min or TGF-β (2 ng/ml) for 15 min. Membranes were incubated with anti-phospho-Smad2 (Ser465/467) (1:1000) and then followed by incubation with peroxidase labelled anti-rabbit IgG (1:2000) and ECL detection. Anti-GAPDH was used as a loading control. Normalized data in each case are shown as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA fooled by least significant difference post-hoc analysis. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with untreated control.

phosphorylation with a peak of 3.5-fold ($p < 0.01$) observed at 60 min (Fig. 4C). Taken together, these results demonstrate that exposure of VSMCs to endogenous and exogenous sources of ROS can trigger Smad dependent TGFBR1 signalling pathways.

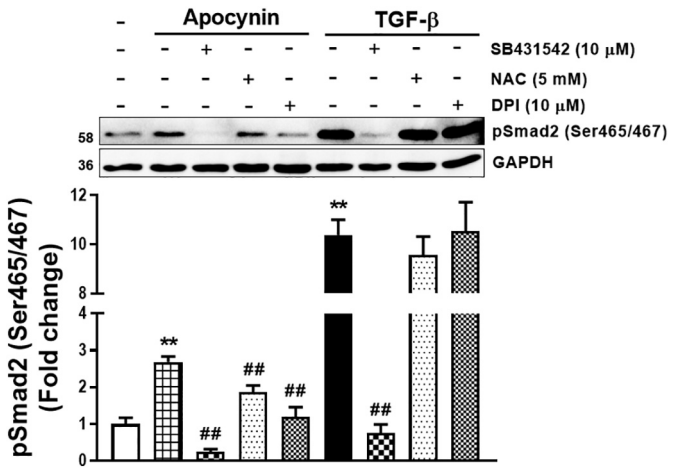

Fig. 5. Apocynin mediated phosphorylation of Smad2 at carboxy terminal is a ROS/Nox dependent pathway in human VSMCs. VSMCs were stimulated with apocynin (10 μM) for 2 h or TGF-β (2 ng/ml) for 15 min in the presence and absence of the TGFBR1 antagonist SB431542 (10 μM), ROS scavenger NAC (5 mM) or Nox inhibitor DPI (10 μM). Membranes were incubated with anti-phospho-Smad2 (Ser465/467) (1:1000) and then followed by incubation with peroxidase labelled anti-rabbit IgG (1:2000) and ECL detection. Anti-GAPDH was used as a loading control. Normalized data in each case are shown as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA fooled by least significant difference post-hoc analysis. $^{**}p < 0.01$ compared with untreated control, $^{\#\#}p < 0.01$ compared with agonists.

3.5. Apocynin mediated ROS production phosphorylates Smad2 carboxy terminal in human VSMCs

ROS mediated TGFBR1 signalling stimulates Smad pathways but little is known by what means ROS may regulate this signalling pathway. Here we investigated whether or not apocynin stimulation of Smad2 phosphorylation is occurring via direct or indirect TGFBR1 activation. VSMCs were treated with apocynin for 2 h in the presence and absence of the selective TGFBR1 antagonist SB431542 [38]. Treatment of VSMCs with apocynin stimulated Smad2 phosphorylation to 2.5-fold ($p < 0.01$) (Fig. 5) and in the presence of SB431542 this response was completely inhibited (lane 2 vs lane 3)($p < 0.01$). To identify the role of the source of ROS involved in apocynin mediated Smad2 phosphorylation, ROS scavenger NAC and non-selective Nox inhibitor, DPI were utilized. Treatment with NAC partially inhibited apocynin mediated Smad2 phosphorylation and in the presence of DPI this response was completely attenuated ($p < 0.01$) (Fig. 5). TGF-β treated VSMCs stimulated Smad2 phosphorylation was completely inhibited in the presence of SB431542 (Fig. 5). ROS/Nox antagonists NAC and DPI had no effect on TGF-β mediated Smad2 phosphorylation. Taken together, these results show that apocynin mediated Smad2 phosphorylation is occurring via the TGFBR1 and is dependent on Nox activity acting upstream of TGBFR1. However, as expected these agents had no effect on the direct TGF-β mediated Smad2 phosphorylation.

3.6. Apocynin mediated ROS production activates ROCK signalling in human VSMCs

Several studies demonstrate that ROS can activate Rho/ROCK pathways [39–41] and ROCK signalling pathways is also involved in TGFBR1 activation [14,42]. As an example we have shown that Rho/ROCK is involved in GPCR mediated transactivation of TGFBR1 [13,22,43]. To investigate whether or not apocynin was signalling via ROCK dependent pathways, we measured the immediate downstream product of ROCK, phosphorylated ezrin/radixin/moesin (pERM). VSMCs treated with apocynin (10 μM) for 15 min stimulated the

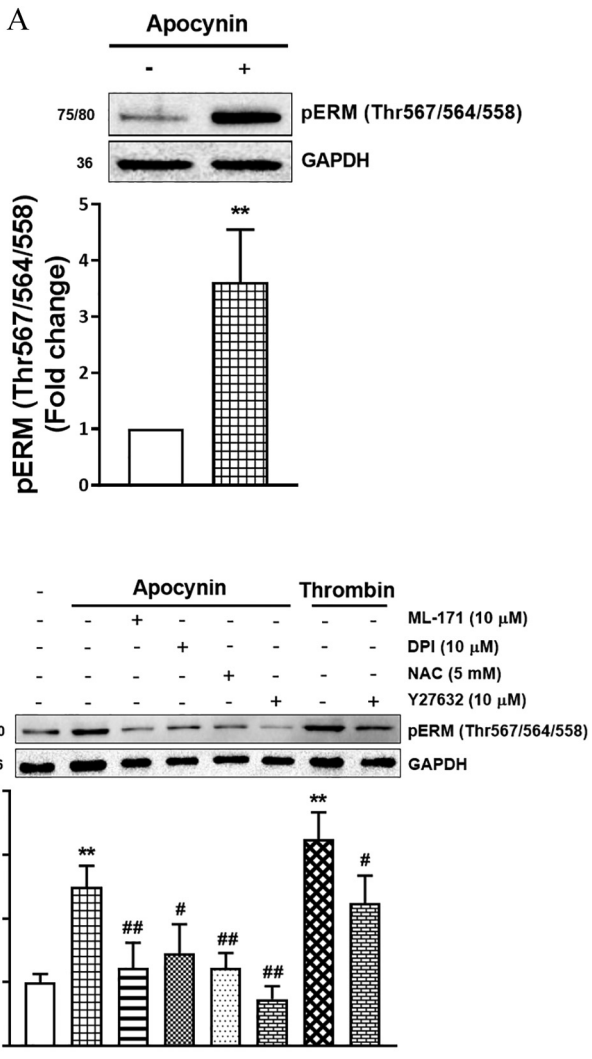

Fig. 6. Apocynin mediated ROS production activates ROCK signalling in human VSMCs. (A) VSMCs were stimulated with apocynin (10 μM) for 15 min. (B) VSMCs were stimulated with apocynin (10 μM) for 15 min or thrombin (10 IU/ml) for 5 min in the presence and absence of the Nox1 inhibitor ML-171 (10 μM), Nox inhibitor DPI (10 μM), ROS scavenger NAC (5 mM) or ROCK inhibitor Y27632 (10 μM) or with thrombin (10 IU/ml) in the presence and absence of ROCK inhibitor Y27632 (10 μM). Membranes were incubated with anti-phospho-Ezrin(Thr567)/Radixin(Thr567)/Moesin(Thr558) (1:2000) and then followed by incubation with peroxidase labelled anti-rabbit IgG (1:2000) and ECL detection. Anti-GAPDH was used as a loading control. Normalized data in each case are shown as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA fooled by least significant difference post-hoc analysis. $^{**}p < 0.01$ compared with untreated control, $^{\#}p < 0.05$ and $^{\#\#}p < .01$ compared with agonists.

phosphorylation of ERM to 3.6-fold ($p < 0.01$) as compared to non-treated controls (Fig. 6A). Apocynin mediated ERM phosphorylation was completely inhibited in the presence of Nox inhibitors, ML-171 ($p < 0.01$) and DPI ($p < 0.05$), and the ROS scavenger NAC ($p < 0.01$) (Fig. 6B). ROCK inhibitor Y27632 completely inhibited apocynin stimulated phospho-ERM (Fig. 6B). Treatment with GPCR agonist thrombin used as a control, showed elevated ERM phosphorylation which was inhibited by the respective antagonist (Fig. 6B). Together these results show that apocynin mediated ROS production stimulates ROCK signalling pathways in VSMCs.

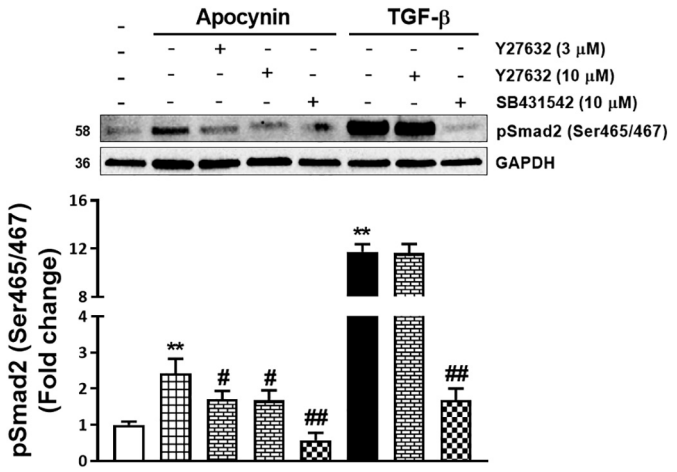

Fig. 7. Apocynin mediated phosphorylation of Smad2 at carboxy terminal is a ROCK dependent pathway in human VSMCs. VSMCs were stimulated with apocynin (10 μM) for 2 h or TGF-β (2 ng/ml) for 15 min in the presence and absence of the ROCK inhibitor Y27632 (3 μM and 10 μM) or TGFBR1 antagonist SB431542 (10 μM). Membranes were incubated with anti-phospho-Smad2 (Ser465/467) (1:1000) and then followed by incubation with peroxidase labelled anti-rabbit IgG (1:2000) and ECL detection. Anti-GAPDH was used as a loading control. Normalized data in each case are shown as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA fooled by least significant difference post-hoc analysis. $^{**}p < 0.01$ compared with untreated control, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with agonists.

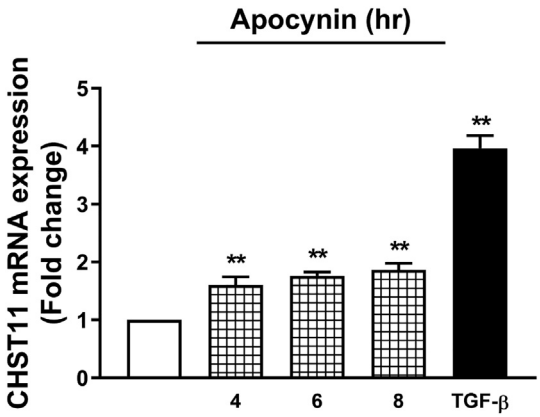

Fig. 8. Apocynin mediated CHST11 mRNA expression in human VSMCs. VSMCs were stimulated with apocynin (100 μM) for 4, 6 and 8 h or TGF-β (2 ng/ml) for 4 h. Total RNA was harvested and mRNA of CHST11 was analyzed using qRT-PCR. 18S was used as a house keeping gene. Results are expressed as mean ± SEM from four independent experiments and statistical significance was determined by One-way ANOVA followed by least significant difference post-hoc analysis. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with untreated control.

3.7. Apocynin via ROCK dependent pathways leads to phosphorylation of Smad2 carboxy terminal

GPCR transactivation dependent signalling via cytoskeletal rearrangement and ROCK dependent pathways can transactivate the TGFBR1 pathway to stimulate Smad signalling [9,13,14,44]. Having shown that apocynin activates ROCK dependent pathways (Fig. 6), we utilized the ROCK inhibitor, Y27632, to investigate the role of ROCK in apocynin mediated Smad2 phosphorylation.

Treatment of VSMCs with apocynin stimulated Smad2 phosphorylation to 2.4-fold ($p < 0.01$) and this response was inhibited by 50% ($p < 0.05$) in the presence of 3 μM and 10 μM of Y27632 and completely inhibited ($p < 0.01$) by SB431542 (Fig. 7). TGF-β stimulated Smad2 phosphorylation was unaffected by ROCK inhibitor and as expected was completely inhibited in the presence of the TGFBR1 antagonist. This data demonstrates apocynin stimulates the TGFBR1 and Smad signalling via ROCK dependent pathways.

3.8. Apocynin mediated ROS production stimulates the mRNA expression of CHST11 in human VSMCs

Apocynin via Nox dependent pathways stimulates ROS and activates key elements which activate the TGFBR1/Smad signalling cascade. We were interested in investigating whether apocynin stimulation of this pathway produces sufficient cellular activation to induce phenotypic change as evidenced by altered gene expression. In VSMCs TGF-β is pro-atherogenic through its actions on the elongation of glycosaminoglycan (GAG) chains on proteoglycans [45–47]. In this context we explored the expression of the rate limiting enzyme involved in GAG chain elongation CHST11 [16,48–50]. VSMCs were treated with apocynin (100 μM) for 4, 6 and 8 h or TGF-β (2 ng/ml) for 4 h. Treatment with apocynin increased the expression of CHST11 to 1.6-fold ($p < 0.01$) at 4 h this stimulation was observed for up to 8 h ($p < 0.01$). TGF-β used as a control mediated a 3.9-fold ($p < 0.01$) increase in the expression of CHST11 (Fig. 8A). This data indicates that apocynin activated ROS signalling pathways stimulating TGFBR1 can produce a *bone fide* cellular response by stimulating the mRNA expression of CHST11.

4. Discussion

ROS is a complex second messenger generated by the actions of many growth factors and hormones, therefore the exploitation of the signalling pathways of endogenous ROS production has allowed us to identify a role for ROS in the activation of TGFBR1 without the activation of multiple pathways. We have demonstrated that apocynin activates Nox and increases ROS levels in human VSMCs leading to phosphorylation of the TGFBR1. ROS stimulates the phosphorylation of TGFBR1 and subsequently activates canonical TGFBR1 signalling leading to an increase in the formation of carboxyl-terminal phosphorylated Smad2. Apocynin stimulated ROS production leads to the activation of ROCK signalling pathways which mediates TGFBR1 signalling. Apocynin mediated Nox-dependent ROS production upregulates the mRNA expression of CHST11 demonstrating that activating ROS signalling pathways is sufficient to stimulate gene expression in human VSMCs.

The proposed molecular mechanism of the pharmacological action (s) of apocynin is not fully understood. Apocynin is involved in the impairment of Nox activation [18] and has been shown to act as a ROS scavenger [21]. For apocynin to exert its actions as a Nox inhibitor it needs to be activated by myeloperoxidase (MPO) and hydrogen peroxide to generate an apocynin dimer which inhibits Nox [51,52]. In HEK293 cells which do not express MPO, apocynin mediated dimer formation did not occur however when treated with human derived MPO apocynin dimer formation was detected [21]. MPO is present in phagocytic cells but absent in non-phagocytic cells such as VSMCs and endothelial cells [53,54]. In vascular fibroblasts, apocynin mediated ROS production was formed from superoxide [17]. Interestingly, in our VSMCs model apocynin mediated ROS production was inhibited by broad Nox inhibitor DPI and specific Nox1 antagonist ML-171 indicating that Nox1 is the source of apocynin stimulated ROS production. Nox1 and Nox2 mainly generate superoxide ROS [33]. However, in monocyte-like cells apocynin induces hydrogen peroxide [55]. Thus, apocynin is an inducer of ROS in non-phagocytic cells independent of the cell type.

Apocynin via Nox 1 stimulates ROS to activate TGFBR1 signalling. Nox1 is involved in the control of cytoskeletal organization and cellular migration in transformed kidney fibroblasts [56]. A family of small Rho GTPases and their downstream target, ROCK, modulate cytoskeletal

reorganization [13,57–60]. ROCK signalling is involved in GPCR mediated transactivation of the TGFBR1 [13,14]. In mouse lung epithelial cells, thrombin and lysophosphatidic acid stimulated TGFBR1 signalling is dependent on ROCK and integrin signalling pathways [61]. In VSMCs, thrombin stimulated Smad2 carboxy terminal phosphorylation is inhibited by ROCK antagonist, thus demonstrating a role for ROCK signalling in TGFBR1 activation [13]. We show that endogenous production of ROS by apocynin stimulates ROCK signalling pathways which stimulates TGFBR1 pathways in VSMCs. The role of ROCK is well characterised in GPCR transactivation of the TGFBR1 [14,16,22], thus this work identifies a potential role of ROS in transactivation of the TGFBR1.

GPCRs are involved in transactivation of PTKR and S/TKR pathways [13,16,62–64]. However, most GPCR agonists generate ROS which are confounding in other signalling pathways. Using apocynin as a ROS agonist we investigated the role of ROS and Nox in activating the TGFBR1 in the absence of the activation of concurrent growth factor signalling pathways. In VSMCs treated with TGF-β there is a marked increase in GAG elongation on the proteoglycan, biglycan, [8] accompanied by an increase in the genes involved in GAG chain elongation [49,50]. Thrombin via transactivation of the TGFBR1 is involved in GAG chain elongation [8,13] and GAG gene expression [16,25]. Collectively these results show that the TGF-β signalling pathways are involved in GAG gene expression and functional GAG elongation [23]. Similarly, apocynin mediated TGFBR1 activation resulting in the up-regulation of the GAG synthesizing enzyme expression. Thus, increasing Nox-dependent ROS level is sufficient to generate *bone fide* cellular responses such as GAG chain elongation.

Apocynin is widely used in studies of inflammation [65], atherosclerosis [66], neurodegenerative conditions [67] and in lung injury [68]. Apocynin dimerization occurs in the presence of MPO [51,52], non-phagocytic cells lack the expression of this enzyme [17], therefore, the application of apocynin in an in vivo model will differ to its use in vitro. In an animal model of atherosclerosis, apocynin is used as a Nox inhibitor [66,69], however, the situation is different from an in vitro setting as phagocytic cells in the vessel wall will produce and release MPO which will be taken up by the non-phagocytic cells. In an in vitro setting apocynin is a powerful tool to investigate the role of ROS dependent signalling pathways. Studying ROS production in the absence of growth factor activated signalling pathways has allowed us to directly study the mechanisms involved in ROS mediated TGFBR1 activation. The identification of Nox/ROS pathways in activating TGF-β mediated Smad signalling allows future signalling studies to investigate the role of ROS in GPCR transactivation of the TGFBR1. An established role for ROS in GPCR transactivation of the EGFR and this newly emerging data for potential involvement of ROS in TGFBR1 transactivation identified the importance of a deeper understanding of the role of ROS in GPCR transactivation dependent signalling and as such ROS would represent the first common mechanism and hence the first potential target to prevent all transactivation dependent signalling.

In conclusion, this work shows that apocynin can stimulate Nox leading to the production of ROS. In other cell types apocynin inhibits Nox and ROS production. The outcome is completely dependent upon the presence and absence of MPO. In the context of apocynin stimulated Nox and increased ROS we have shown that ROS alone is enough to stimulate gene expression. These results impact on the use of apocynin in pharmacological and therapeutic studies.

Funding

RM was supported by the University of Mosul, Iraq. Support was received from the University of Queensland through a personal support package to PJL and by the University of Queensland Early Career Grant (DK) (Grant no. 1832825). DK is supported by National Health and Medical Research Council-Peter Doherty (1160925) and National Heart Foundation (102129) Fellowships.

Declaration of Competing Interest

The authors declare that there is NO Conflict of Interest associated with this paper.

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