Protective role of epigallocatechin-3-gallate in NADPH oxidase-MMP2-Spm-Cer-S1P signalling axis mediated ET-1 induced pulmonary artery smooth muscle cell proliferation

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Abstract

The signalling pathway involving MMP-2 and sphingosine-1-phosphate (S1P) in endothelin-1 (ET-1) induced pulmonary artery smooth muscle cell (PASMC) proliferation is not clearly known. We, therefore, investigated the role of NADPH oxidase derived $O_2^{\cdot-}$ -mediated modulation of MMP2-sphingomyelin-ceramide-S1P signalling axis in ET-1 induced increase in proliferation of PASMCs. Additionally, protective role of the tea catechin, epigallocatechin-3-gallate (EGCG), if any, in this scenario has also been explored. ET-1 markedly increased NADPH oxidase and MMP-2 activities and proliferation of bovine pulmonary artery smooth muscle cells (BPASMCs). ET-1 also caused significant increase in sphingomyelinase (SMase) activity, ERK1/2 and sphingosine kinase (SPHK) phosphorylations, and S1P level in the cells. EGCG inhibited ET-1 induced increase in SMase activity, ERK1/2 and SPHK phosphorylations, S1P level and the SMC proliferation. EGCG also attenuated ET-1 induced activation of MMP-2 by inhibiting NADPH oxidase activity upon inhibiting the association of the NADPH oxidase components, p47phox and p67phox in the cell membrane. Molecular docking study revealed a marked binding affinity of p47phox with the galloyl group of EGCG. Overall, our study suggest that ET-1 induced proliferation of the PASMCs occurs via NADPH oxidase-MMP2- Spm- Cer-S1P signalling axis, and EGCG attenuates ET-1 induced increase in proliferation of the cells by inhibiting NADPH oxidase activity.

Keywords EGCG · MMP-2 · NADPH oxidase · proMMP-2 · SMase · SPHK · S1P

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Introduction

Pulmonary artery hypertension (PAH) is a devastating life threatening disorder characterized by elevated pulmonary arterial pressure, which results in right ventricular hypertrophy (Chaumais et al. 2015; Stenmark et al. 2009). A considerable number of reports have suggested that ET-1 plays an important role in producing a variety of pulmonary diseases, for example, PAH via membrane bound ET receptors (Chakraborti et al. 2015; Kim et al. 2015). The receptors that mediate biological effects of ET-1 are ET_A and ET_B and these are found in vascular SMCs (Chakraborti et al. 2015; Khalil 2011; Chen et al. 2009).

MMP-2 is known to regulate remodelling of pulmonary vasculature and its dysregulation promotes development of a variety of lung diseases like PAH (Stenmark and Mecham 1997; Stamenkovic 2003; Chelladurai et al. 2012). MMP-2 has also been suggested to play a crucial role for increase in

cell proliferation (He et al. 2007). In some cell types, ET-1 markedly induces MMP-2 activation, which has been suggested to influence remodelling of tissues (He et al. 2007).

Oxidative stress activates several enzymes, which alone and in combination, alters different cellular responses. In vascular cells, the predominant pathway for $O_2^{\cdot-}$ generation occurs via NADPH oxidase dependent mechanism (Lambeth 2004; Lassegue and Clempus 2003). Stimulation of NADPH oxidase derived $O_2^{\cdot-}$ production in pulmonary artery is known to be an important event in the pathogenesis of a variety of lung diseases, for example, PAH (Wedgwood and Black 2003). $O_2^{\cdot-}$ and its derivatives such as peroxy and hydroxyl radicals have been demonstrated to activate proMMP-2 in different systems (Chakraborti et al. 2003; Mandal et al. 2005, 2004).

Sphingosine-ceramide-sphingosine 1-phosphate (Spm-Cer-S1P) pathway has been suggested to play an important role in SMC proliferation (Augé et al. 2004; Maupas-Schwalm et al. 2004). In this pathway, neutral sphingomyelinase converts sphingomyelin to ceramide, which then forms S1P by the enzyme sphingosine kinase. A discernible increase in S1P level has been shown to be associated with PAH (Chen et al. 2014). However, no evidence is currently available on the role of MMP-2 and Spm-Cer-S1P pathway in ET-1 induced increase in proliferation of pulmonary artery smooth muscle cells.

Tea contain polyphenols importantly catechins, of which epigallocatechin gallate (EGCG) is known to be the most biologically active compound and received special attention as prospective dietary intervention in vascular diseases (Chowdhury et al. 2016). EGCG has been shown to inversely associate with SMC migration and proliferation (Wang et al. 2014). Due to its role in inhibiting different ROS-mediated signalling pathways, EGCG attenuates progression of cardiac hypertrophy (Peng et al. 2010).

NADPH oxidase found in cells that transfers reducing equivalents from NADPH to oxygen resulting $O_2^{\cdot-}$ production. NADPH oxidase consists of membrane associated 91 kDa (gp91phox) and 22kDa (p22phox) subunits, and three cytosolic proteins p47phox, p67phox and a small GTPase Rac. Upon stimulation, the cytosolic components are translocated to the cell membrane for their assembly and to complex with gp91phox and p22phox to an active assembled form (Meyer and Schmitt 2000; Chakraborti et al. 2009). Apocynin, a well known inhibitor of NADPH oxidase, is known to prevent association of the p47phox and p67phox subunit of NADPH oxidase complex in the membrane, and inhibits $O_2^{\cdot-}$ production (Meyer and Schmitt 2000).

Herein, we determined the role of NADPH oxidase-MMP2-Spm-Cer-S1P pathway in ET-1 induced increase in proliferation of bovine PASMCs, and the role of the galloyl group containing catechins, EGCG in this scenario. We have also determined the role of EGCG on ET-1 induced increase in the association between p47phox and p67phox in the SMC

membrane, and ascertained the binding of p47phox with EGCG by molecular modelling and docking studies. Our study suggests that EGCG by inhibiting NADPH oxidase-MMP2-Spm-Cer-S1P signalling axis attenuates ET-1 induced increase in proliferation of BPASMCs.

Materials and methods

Chemicals

BPASMCs were obtained from Cell Applications (San Diego, CA). Fetal calf serum (FCS), ET-1, GM6001, EGCG, EGC, GW4869 and PD98059 were the products of Sigma-Aldrich (St. Louis, MO, USA). MMP-2 inhibitor-I, monoclonal anti p47phox and monoclonal anti p67phox were obtained from Santa Cruz (Texas, USA). SKI-I was the product from Abcam (Cambridge, MA, USA). Polyclonal anti-MMP-2 antibody was obtained from Chemicon International Inc. (Temecula, CA, USA). Peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Zymed laboratories (San Francisco, CA, USA). Bichinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). ET_A, ET_B, MMP-2, ERK-1, ERK-2, SMase, SPHK and scrambled siRNA duplexes were obtained from Integrated DNA Technologies (IDT) (San Jose, CA, USA) and also from Invitrogen (Carlsbad, CA, USA). Lipofectamine was the product of Invitrogen (Carlsbad, CA, USA).

Cell culture

BPASMCs were cultured in tissue culture flasks using DMEM supplemented with FCS (10%) and penicillin–

Table 1 SiRNA duplexes of ET_A, ET_B, MMP-2, SMase, SPHK, ERK-1 and ERK-2

ET _A siRNA	5'-UGACUUGUGAGAUGUUAACCGAAG-3' and 5'-CUUCGGUUUAACAUCUCACAAGUCAUG-3'
ET _B siRNA	5'-AGAAGAGUGGUAGCAAUUGCCTT-3' and 5'-AAGGCAAUUUGCAUACCACUCUUCUUU-3'
MMP-2 siRNA	5'-GAACCAGAUCAUACAGG-3' and 5'-CUUGGUCUAGUGUAUGUCC-3'
SMase siRNA	5'-ACCCACCACCUACGAGAAGCGCCAG-3' and 5'-CUGGCGCUUCUCGUAGGUGGUGGUAU-3'
SPHK siRNA	5'-UGGAGAAAGGCAGGCACAUGGAGUG-3' and 5'-CACUCCAUGUGCCUGCCUUUCUCCAUG-3'
ERK-1 siRNA	5'-GACAGACCUGUACAAAUUGCUCAAA-3' and 5'-UUUGAGCAAUUUGUACAGGUCUGUCUC-3'
ERK-2 siRNA	5'-GCUACACCAAUCUCUCGUACAUCGG-3' and 5'-CCGAUGUACGAGAGAUUGGUGUAGCGC-3'

(obtained from IDT, San Jose, CA, USA)

Table 2 Primer pairs for cDNA amplifications of ET_A, ET_B, MMP-2, SMase, SHPK, ERK-1, ERK-2 and β -actin

	Forward primer	Reverse primer
ET _A receptor	ACATTGCCATTACCTTCG	ACTGATGACACCTCCAACCA
ET _B receptor	CTGAGGCTGTGGGTTTGTAT	AACAATGCAGTGATGGCCAG
MMP-2	TCTTACGACAGTTGCACCAC	CACACCACAACCTTCCCATCA
SMase	TTTGACATCATCTGCGGAGA	ACTCTCCAAGACCTTTTGCA
SHPK	TGCTGGTGTGTAAATCCG	ATCTCCGGACATGACCACCA
ERK-1	CCTTTGAGCATCAGACCTAC	CAGGATCTGGTACAGGAAGT
ERK-2	AGTACAGGACCTCATGGAAA	CTCTGTCAAGAACCCTGTGT
β -actin	GAGCGGGAATCGTCCGTGAC	GTGTTGGCGTAAGGTCCTTGC

streptomycin (2%) in a CO₂ (5%) containing humidified chamber maintained at 37°C. All experiments were performed in serum free media and the cells were used between passages 5 and 10.

Cell proliferation assay

Colorimetric MTT assay was employed to determine proliferation of the smooth muscle cells. MTT is metabolized by

Fig. 1 Effect of ET-1 on proliferation of BPASMCs. **a** Effect of ET_A and ET_B siRNA transfection on ET_A and ET_B mRNA expression in the cells. **b** Effect of ET_A and ET_B siRNA transfection on ET_A and ET_B protein expression in the cells. **c** Dose-response profile of ET-1 induced proliferation of BPASMCs. **d** Effect of bosentan (ET-1 receptor antagonist) on ET-1 induced cell proliferation. **e** Effect of ET_A and ET_B

siRNA transfection on ET-1 induced cell proliferation. ET_A and ET_B siRNA duplexes (presented in Table 1) were obtained from IDT, San Jose, CA, USA. β -actin was used as the loading control. Densitometric profiles are also shown. Results are mean $\pm$ SE ($n = 4$). ^a $p < 0.05$ compared with basal condition; and ^b $p < 0.05$ compared with ET-1 treatment

NAD dependent dehydrogenase and forms a coloured product (formazan), which was measured at a wavelength of 562 nm.

Assay of NADPH oxidase derived $O_2^{\cdot -}$ formation

NADPH oxidase activity was determined by measuring $O_2^{\cdot -}$ generation by SOD inhibitable cytochrome c reduction assay (Chakraborti et al. 2017).

Gelatin Zymography

Gelatinase activities of the samples were analyzed by substrate-gel electrophoresis (zymography) using 0.1% gelatin as previously described (Das et al. 2004).

Western blot

Western blot was performed by following the procedure described by Towbin (Towbin et al. 1979).

Assay of sphingomyelinase activity

Sphingomyelinase (SMase) activity was assayed using SMase activity assay kit (Echelon, Salt Lake City, USA) according to the manufacturer's protocol.

Assay of S1P level

S1P level was determined using S1P assay kit (Echelon, Salt Lake City, USA) according to the manufacturer's protocol.

Transfection of siRNA

ET_A and ET_B receptors, MMP-2, ERK-1, ERK-2, SMase and SPHK siRNA transfections to the cells were done with lipofectamine reagent as previously described (Sarkar et al. 2016). The siRNA duplexes described in Table 1 (obtained from IDT, San Jose, CA, USA) and Table- ST1 (obtained from Invitrogen, Carlsbad, CA, USA), respectively, were used in the present study.

Fig. 2 Role of Spm–Cer–S1P pathway on ET-1-induced cell proliferation. **a** Effect of SMase and SPHK siRNA transfection to the cells on SMase and SPHK mRNA expression. **b** Effect of SMase and SPHK siRNA transfection on SMase and SPHK protein expression. **c** Effect of pretreatment with GW4869 (sphingomyelinase inhibitor) and SKI-I (SPHK inhibitor) on ET-1 induced cell proliferation. **d** Effect of SMase and SPHK siRNA transfection on ET-1 induced cell proliferation. **e** Time-response profile of ET-1 induced increase in SMase activity. **f** Effect of pretreatment with bosentan, GW4869 and SKI-I on ET-1 induced SMase activity in the cell lysate. **g** Effect of ET_A, ET_B, SMase and SPHK siRNA transfection to the cells on ET-1 induced SMase activity in the cell lysate. **h** Time-response profile of ET-1 effect on S1P level in the cell lysate. **i** Effect of pretreatment with bosentan, GW4869 and SKI-I on ET-1 induced S1P level in the cell lysate. **j** Effect of ET_A, ET_B, SMase and SPHK siRNA transfection to the cells on ET-1 induced S1P level in the cell lysate. **k** Time-response profile on ET-1 induced SPHK phosphorylation. **l** Effect of pretreatment with bosentan, GW4869 and SKI-I on ET-1 induced SPHK phosphorylation. **m** Effect of ET_A, ET_B, SMase and SPHK siRNA transfection on ET-1 induced SPHK phosphorylation. ET_A, ET_B, SMase and SPHK siRNA duplexes (presented in Table 1) were obtained from IDT, San Jose, CA, USA. β -actin was used as the loading control. Densitometric profiles are also shown. Results are mean $\pm$ SE ($n = 4$). ^a $p < 0.05$ compared with basal condition; and ^b $p < 0.05$ compared with ET-1 treatment

RNA isolation and semiquantitative RT-PCR analysis

Total RNA was isolated with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Semiquantitative RT-PCR analyses were performed using the primer pairs for ET_A, ET_B, MMP-2, ERK-1, ERK-2, SMase and SPHK (in the 5'–3' direction) (Table 2) by following the procedure previously described (Sarkar et al. 2016).

Estimation of protein

Proteins were estimated by BCA protein assay reagent using bovine serum albumin (BSA) as the standard (Smith et al. 1985).

Modelling and docking studies

Primary sequence of human NADPH oxidase p47phox subunit (1–390 amino acids) was downloaded from Uniprot (ID: P14598), and subjected to template-based threading and

Table 3 List of inhibitors with their properties

Inhibitor name	Properties	References
Bosentan	Endothelin receptor inhibitor	Chaumais et al. 2015
GW4869	SMase inhibitor	Bautista-Pérez et al. 2015
SKI-I	SPHK inhibitor	Eisinger and Ammer 2009
GM6001	MMP general inhibitor	Martin-Martin et al. 2011
MMP-2 inhibitor-I	MMP-2 specific inhibitor	Garanich et al. 2005
PD98059	ERK1/2 inhibitor	Chen et al. 2014
Apocynin	NADPH oxidase inhibitor	Mora-Pale et al. 2009

modelling server I-TASSER for full length structure modelling (Roy et al. 2010). The final structure of p47phox subunit was further refined by Smart Minimizer of Discovery Studio (DS) 2.5 with RMS gradient of 0.1 and its stereochemistry was validated through SAVES server <http://services.mbi.ucla.edu/SAVES>. Full length p47phox subunit contains mainly the N-terminal PX domain, SH3 domain and the C-terminal p47phox domain. Structures of green tea catechin EGCG (galloyl group containing) and EGC (devoid of galloyl group) were downloaded from Ligand Expo (Feng et al. 2004) and PubChem (Kim et al. 2016) databases. The catechins were prepared at pH 7.4 with salt concentration 0.1 M and their respective tautomers & isomers were generated. Finally, the catechins were allowed to dock at the reported binding site of p47phox near Cys378 (Mora-Pale et al. 2009; Yu et al. 2008; Kawahara and Lambeth 2007). Genetic Optimization for Ligand Docking (GOLD 5.2) software was used for docking with a defined binding cavity of 20 Å around the residue Cys378 (Jones et al. 1997). Ten binding poses for each isomeric/tautomeric ligand conformation were generated for both the catechins and were ranked on the basis of CHEMPLP fitness score (Chakraborti et al. 2018). The selected poses were further minimized in Smart Minimizer of DS 2.5 with RMS gradient of 0.1 followed by the calculation of their binding-free energy (dG) and interaction energies (IEs). The computational methods have been discussed in detail in our previous works (Bhuyan et al. 2015; Beswick et al. 2001; Newby 2006).

Statistical analysis

Data were analyzed by unpaired “*t*” test and analysis of variance (ANOVA) followed by the test of least significant differences for comparisons within and between the groups, and $p < 0.05$ was considered significant (David 1978).

Results

Role of ET-1 on proliferation of PSMCs

Transfection of the cells with two distinct sets of ET_A and ET_B siRNAs markedly inhibited mRNA and protein expression of ET_A and ET_B receptors, respectively (Fig. 1a and b; S1A & B). Treatment of the SMCs with ET-1 increases the cell proliferation in a dose-dependent manner and 10 nM was determined to be the optimum concentration (Fig. 1c). Pretreatment of the cells with bosentan (Table 3) prevented ET-1 induced proliferation of the cells, which indicates that the effect of ET-1 on the cell proliferation was mediated via ET-1 receptor (Fig. 1d). Transfection of the cells with two distinct sets of ET_A siRNAs, but not ET_B siRNAs, inhibited ET-1 induced increase in the cell proliferation (Fig. 1e; S1C), which suggests that proliferation of the cells occurs through ET_A receptor mediated pathway.

Role of Spm-Cer-S1P axis in ET-1 induced cell proliferation

Transfection of the cells with two distinct sets of SMase and SPHK siRNAs inhibited SMase and SPHK mRNA and protein expression, respectively (Fig. 2a and b; S2A&B).

Pretreatment of the cells with GW4869 and SKI-I (Table 3) inhibited ET-1 induced increase in the smooth muscle cell proliferation (Fig. 2c). Transfection of the cells with two distinct sets of SMase and SPHK siRNA inhibited ET-1 induced increase in the cell proliferation (Fig. 2d; S2C).

ET-1 treatment to the cells resulted in an increase in SMase activity, S1P level and SPHK phosphorylation in a time dependent manner and the optimum time has been determined to be 6 h (Fig. 2e, h and k).

Pretreatment of the cells with bosentan and GW4869, but not SKI-I, inhibited ET-1 induced increase in the SMase activity (Fig. 2f). Transfection of the cells with two distinct sets of ET_A and SMase siRNAs, but not two distinct sets of ET_B and SPHK siRNAs, inhibited ET-1 induced increase in SMase activity (Fig. 2g; S2D). On the other hand, pretreatment of the cells with GW4869 markedly, while bosentan and SKI-I fully, inhibited ET-1-induced increase in S1P level (Fig. 2i). Transfection of the cells with two distinct sets of ET_A, SMase and SPHK siRNAs, but not two distinct sets of ET_B siRNAs, inhibited ET-1 induced increase in S1P level (2J; S2E). Pretreatment of the cells with bosentan and SKI-I, but not GW4869, inhibited ET-1 induced increase in SPHK phosphorylation (Fig. 2l). Transfection of the cells with two distinct sets of ET_A and SPHK siRNAs, but not two distinct sets of ET_B and SMase siRNAs, inhibited ET-1 induced increase in SPHK phosphorylation (Fig. 2m; S2F).

The above results suggest that ET-1 induced cell proliferation occurs via increase in the production of S1P upon ET_A

Fig. 3 Role of MMP-2 and ERK 1/2 in Spm-Cer-S1P signaling pathway on ET-1 induced cell proliferation. **a** Effect of MMP-2, ERK-1 and ERK-2 siRNA transfection on MMP-2, ERK-1 and ERK-2 mRNA expression. **b** Effect of pretreatment with GM6001 (MMP general inhibitor), MMP-2 inhibitor-I and PD98059 (ERK1/2 inhibitor) on ET-1 induced cell proliferation. **c** Effect of MMP-2, ERK-1 and ERK-2 siRNA transfection on ET-1 induced cell proliferation. **d** Effect of pretreatment with MMP-2 inhibitor-I and PD98059 on ET-1 induced SMase activity in the cell lysate. **e** Effect of MMP-2, ERK-1 and ERK-2 siRNA transfection on ET-1 induced SMase activity in the cell lysate. **f** Effect of pretreatment of MMP-2 inhibitor-I and PD98059 on S1P level in the cell lysate. **g** Effect of MMP-2, ERK-1 and ERK-2 siRNA transfection on ET-1 induced S1P level in the cell lysate. **h** Effect of MMP-2 inhibitor-I and PD98059 on ET-1 induced SPHK phosphorylation. **i** Effect of MMP-2, ERK-1 and ERK-2 siRNA transfection on ET-1 induced SPHK phosphorylation. MMP-2, ERK-1, ERK-2 siRNA duplexes (presented in Table 1) were obtained from IDT, San Jose, CA, USA. β -actin was used as the loading control. Densitometric profiles are also shown. Results are mean $\pm$ SE ($n = 4$). ^a $p < 0.05$ compared with basal condition; and ^b $p < 0.05$ compared with ET-1 treatment alone

Fig. 4 Role of ET-1 on ERK1/2 activation in the cells. **a** Effect of ET-1 on ERK1/2 expression and ERK1/2 phosphorylation in the cell lysate. **b** Effect of bosentan, apocynin, PD98059, GW4869, SKI-1 and MMP-2 inhibitor-1 on ET-1 induced ERK1/2 phosphorylation in the cell lysate. β -actin was used as the loading control. Densitometric profiles are also shown. Results are mean $\pm$ SE (n = 4). ^ap < 0.05 compared with basal condition; and ^bp < 0.05 compared with ET-1 treatment

receptor mediated activation of SMase and also SPHK phosphorylation. ET-1 treatment to the cells increases S1P level, which markedly, but not fully, returns to basal level upon

inhibition of SMase activity; however, ET-1 induced increase in S1P level has been observed to be completely attenuated by inhibition of SPHK phosphorylation.

Fig. 5 Role of ET-1 on proMMP-2 expression and activation in the cells. Effect of ET-1 on time response profile of proMMP-2 and MMP-2 (a) activation by gelatin zymography; and (b) protein expression by Western blot in the cell culture supernatant. **c** Time-response profile of

ET-1 induced MMP-2 mRNA expression in the cells. Densitometric profiles are also shown. Results are mean $\pm$ SE (n = 4). ^ap < 0.05; and ^bp < 0.05 compared with basal condition

Role of MMP-2 and ERK1/2 in Spm-Cer-S1P axis mediated ET-1 induced cell proliferation

Transfection of the cells with two distinct sets of MMP-2 and ERK1/2 siRNAs significantly attenuated MMP-2 and ERK1/2 mRNA expression (Fig. 3a; S3A). Pretreatment of the cells with GM6001, MMP-2 inhibitor-I and PD98059 (Table 3) attenuated ET-1 caused cell proliferation (Fig. 3b). Pretreatment of the cells with MMP-2 inhibitor-I, but not PD98059, prevented ET-1 induced increase in SMase activity (Fig. 3d), while pretreatment with MMP-2 inhibitor-I and PD98059 attenuated ET-1 induced increase in S1P production (Fig. 3f). However, pretreatment of the cells with PD98059, but not MMP-2 inhibitor-I, attenuated ET-1 stimulated SPHK phosphorylation (Fig. 3h). Transfection of the cells with two distinct sets of MMP-2 and ERK1/2 siRNAs followed by treatment with ET-1 showed similar pattern of results obtained using their chemical inhibitors (Fig. 3c, e, g and i; S3B,C,D & E).

The above results suggest that ET-1 induced cell proliferation occurs with the involvement of MMP-2 and ERK1/2. Both MMP-2 and ERK1/2 are involved in S1P production; however, MMP-2 elicits its action via activation of SMase, while ERK1/2 shows its action via phosphorylation of SPHK.

Role of different inhibitors on ET-1 induced ERK1/2 activation

ET-1 treatment stimulated ERK1/2 phosphorylation in a time-dependent manner, and 4h was determined to be the optimum time for the ET-1 response (Fig. 4a). Pretreatment of the cells with bosentan, apocynin, PD98059 (Table 3) prevented ET-1 induced increase in ERK1/2 phosphorylation (Fig. 4b). However, pretreatment of the cells with MMP-2 inhibitor-I, GW4869 and SKI-I did not show any discernible change in ET-1 response on ERK1/2 phosphorylation (Fig. 4b).

Role of ET-1 in NADPH oxidase-mediated proMMP-2 expression and activation and the cell proliferation

ET-1 treatment to the cells elicited time dependent increase in proMMP-2 (72 kDa) and MMP-2 (66 kDa) gelatinolytic activity in the cell culture supernatant, and 24 h was determined to be the optimum time for proMMP-2 activation (Fig. 5a). Immunoblot study with the cell culture supernatant revealed that ET-1 time-dependently increased proMMP-2 (72 kDa) and MMP-2 (66 kDa) expression (Fig. 5b). MMP-2 mRNA

Fig. 6 Role of NADPH oxidase derived $O_2^{\cdot -}$ in ET-1 induced proMMP-2 expression and activation and proliferation of the cells. Effect of pretreatment with apocynin on ET-1 induced proMMP-2 and MMP-2 (a) activation; and (b) protein expression in the cell culture supernatant. c Effect of pretreatment with apocynin on ET-1 induced MMP-2 mRNA expression in the cells. β -actin was used as the loading control. Densitometric profiles

are also shown. d Effect of pretreatment with apocynin on ET-1 induced cell proliferation. e Time response profile of the effect of ET-1 on NADPH oxidase activity in the cell lysate. f Effect of apocynin and MMP-2 inhibitor-I on ET-1 induced NADPH oxidase activity in the cell lysate. Results are mean $\pm$ SE (n = 4). ^ap < 0.05 compared with basal condition; and ^bp < 0.05 compared with ET-1 treatment

expression was determined to be up regulated in a time dependent manner during ET-1 stimulation and the optimal expression was determined to be 24 h (Fig. 5c).

Gelatin zymography and western blot study showed that apocynin pretreatment significantly inhibited ET-1 induced increase in MMP-2 expression and activation (Fig. 6a and b). RT-PCR study revealed that apocynin pretreatment markedly inhibited ET-1 induced MMP-2 mRNA expression (Fig. 6c).

Apocynin pretreatment to the cells prevented ET-1 induced proliferation of the SMCs (Fig. 6d).

Treatment of the cells with ET-1 stimulated NADPH oxidase derived $O_2^{\cdot -}$ production in a time dependent manner and 10 min was determined to be the optimum time for NADPH oxidase activation in the SMCs (Fig. 6e).

Pretreatment with apocynin, but not MMP-2 inhibitor-I, inhibited ET-1 induced increase in NADPH oxidase activity in the cells (Fig. 6f).

The above results suggest that ET-1 mediated increase in the cell proliferation occurs via stimulation of NADPH

oxidase derived $O_2^{\cdot -}$ production, which subsequently activates MMP-2 in the cells.

Role of ET-1 in NADPH oxidase-mediated stimulation of SMase activity, S1P level and SPHK phosphorylation

Pretreatment of the SMCs with apocynin markedly inhibited the ET-1 induced increase in SMase activity (Fig. 7a), S1P level (Fig. 7b) and SPHK phosphorylation (Fig. 7c).

Role of EGCG/EGC on ET-1 induced increase in SMase and SPHK activities, S1P level, ERK1/2 phosphorylation and the cell proliferation

Pretreatment of the cells with EGCG, but not EGC, dose-dependently inhibited ET-1 induced increase in the SMCs proliferation. The optimum concentration of EGCG to inhibit the ET-1 induced cell proliferation was determined to be 50 μ M (Fig. 8).

Fig. 7 Role of NADPH oxidase derived $O_2^{\cdot -}$ in ET-1 induced SMase activity, S1P level and SPHK phosphorylation in the cells. Effect of apocynin on ET-1 induced (a) SMase activity, b S1P level and c SPHK phosphorylation the cell lysate. β -actin was used as the loading control. Densitometric profiles are also shown. Results are mean $\pm$ SE (n = 4). ^ap < 0.05 compared with basal condition; and ^bp < 0.05 compared with ET-1 treatment

Fig. 8 Role of EGCG/EGC on ET-1 induced proliferation of the cells. Dose-response profile of the effect of EGCG/EGC on ET-1 induced proliferation of the SMCs. Results are mean $\pm$ SE (n = 4). ^ap < 0.05 compared with basal condition; and ^bp < 0.05 compared with ET-1 treatment

Pretreatment of the cells with EGCG, but not EGC, markedly inhibited ET-1 induced increase in SMase activity, S1P level, SPHK and ERK1/2 phosphorylations (Fig. 9a–d).

Role of EGCG/EGC on ET-1- induced increase in proMMP-2 expression and activation and NADPH oxidase activation

Gelatin zymogram and western blot studies showed that EGCG, but not EGC, attenuates ET-1 induced increase in proMMP-2 activation and expression, respectively (Fig. 10a and b). RT-PCR study showed that EGCG, but not EGC,

pretreatment markedly inhibited ET-1-induced increase in MMP-2 mRNA expression in the cells (Fig. 10c).

Pretreatment of the cells with EGCG markedly inhibited ET-1 induced increase in NADPH oxidase activity, whereas EGC did not show any discernible effect (Fig. 10d).

Role of EGCG/EGC on ET-1 induced association between p47phox and p67phox

Immunoblot study revealed that treatment of the cells with ET-1 showed association between p47phox and p67phox in the cell membrane. Pretreatment of the cells with EGCG, but not

Fig. 9 Role of EGCG/EGC on ET-1-induced SMase activity, SPHK activation, S1P level and ERK 1/2 phosphorylation in the cells. Effect of EGCG/EGC on ET-1 induced (a) SMase activity. b S1P level. c SPHK phosphorylation; and d ERK1/2 phosphorylation in the cell lysate. β -actin was used as the loading control. Densitometric profiles are also shown. Results are mean $\pm$ SE (n = 4). ^ap < 0.05 compared with basal condition; and ^bp < 0.05 compared with ET-1 treatment

Fig. 10 Effect of EGCG/EGC on ET-1-induced expression and activation of proMMP-2, NADPH oxidase activity, and association between p47phox and p67phox in the cells. Effect of pretreatment with EGCG/EGC on ET-1 induced activation and expression of proMMP-2 determined by (a) gelatin zymography; and (b) Western blot; (c) Effect of pretreatment with EGCG/EGC on ET-1 induced MMP-2 mRNA expression in the cells. (d) Effect of pretreatment with EGCG/EGC on

ET-1 induced NADPH oxidase activity in the cell membrane fraction. (e) Coimmunoprecipitation of p47phox with p67phox in the cell membrane fraction isolated upon pretreatment of the cells with EGCG/EGC followed by addition of ET-1. β-actin was used as loading control. Densitometric profiles are also shown. Results are mean ± SE (n = 4). ^ap < 0.05 compared with basal condition; and ^bp < 0.05 compared with ET-1 treatment

EGC, inhibited ET-1 induced association of p47phox with p67phox in the cell membrane (Fig. 10e).

Binding of p47phox subunit of NADPH oxidase with EGCG/EGC

To explore the binding mode of NADPH oxidase inhibition, computational docking was performed by taking phosphoinositide binding of p47phox subunit of NADPH oxidase with the tea catechins, EGC & EGCG. Among EGCG and EGC, the galloyl group containing EGCG inhibits the p47phox at its PX domain more significantly than the EGC. The PX domain of p47phox subunit usually resides in cytosolic part and preferentially binds phosphatidylinositol-3,4-bisphosphate, resulting in increase in membrane affinity. The interaction of catechins with p47phox subunit was characterized by number of hydrogen bonds (H-bond), π -interaction, interaction energies (IE) in terms of electrostatic (elec) and van der Waals (vdW) interactions (Table 4). The binding free energy (dG), which is a key parameter to study the affinity of compounds, was calculated to be -33.53 kcal/mol for EGCG;

whereas for EGC, it was witnessed only -20.95 kcal/mol (Fig. 11a). Similarly, a considerable difference in interaction energy between p47phox subunit-EGCG/EGC was also observed, where the electrostatic interaction prevailed than the hydrophobic/van der Waals interactions (Fig. 11b). The residues that participate with the interaction with catechins form seven H-bonds, two π - π , and 388 neighbouring contacts with galloyl group containing EGCG. Among them, Tyr24, Tyr26, Asp82, Gly83, Gln84, Arg90 are important in contributing various interactions with EGCG (Fig. 11c). On the other hand, EGC procured only 260 atomic contacts, four H-bonds and one π - π interaction (Fig. 11d). The 3D representation of p47phox subunit-EGCG/EGC interaction is represented in Fig. 11e and f.

Discussion

The present study suggests that ET-1 stimulates proliferation of BPASMCs by inducing MMP2-mediated Spm-Cer-S1P signalling pathway, and that has been observed to be inhibited

Table 4 Key residues of p⁴⁷phox interacting with EGCG/EGC

p ⁴⁷ phox-EGCG	p ⁴⁷ phox-EGC
PHE14	PHE14
SER21	
<u>GLN22</u>	GLN22
HIS23	HIS23
<u>TYR24</u>	TYR24
VAL25	VAL25
TYR26	TYR26
ARG43	ARG43
PHE44	PHE44
THR45	THR45
	TRP80
PHE81	PHE81
<u>ASP82</u>	ASP82
<u>GLY83</u>	GLY83
<u>GLN84</u>	GLN84
ARG85	ARG85
ALA86	ALA86
ALA87	ALA87
GLU88	GLU88
ARG90	ARG90
ASP151	
ILE152	

Residues that form hydrogen bonds are underlined; that take participate in π - π interaction are mentioned as italics; that contribute interaction energy < -4 kcal/mol are marked as bold

upon pretreatment with EGCG. We also observed that EGCG attenuates ET-1 induced increase in proMMP-2 expression and activation by inhibiting NADPH oxidase activity.

An important aspect in pulmonary vascular diseases is the dysregulation in proMMP-2 activation. O₂⁻ has been shown to play a crucial role in proMMP-2 expression and activation (Mandal et al. 2005, 2004). The present study suggests that ET-1 augments O₂⁻ production via ET(R)-NADPH oxidase dependent mechanism. This is evident from the observation that pretreatment of the cells with bosentan (ET receptor antagonist) and apocynin (NADPH oxidase inhibitor) attenuate the response produced by ET-1. ET-1 upon binding to its receptor elicits a plethora of cellular responses by stimulating various intracellular pathways. Our present study suggests that ET-1 markedly increases the SMC proliferation upon activation of ET_A receptor, which is evident from the observation that pretreatment with ET_A siRNA attenuates ET-1 induced increase in the SMC proliferation.

ROS-mediated dysregulation of cell signalling processes have been suggested to play important roles in different types of cardiovascular diseases (Chelladurai et al. 2012). Vascular SMC proliferation has been shown to occur upon stimulation

of NADPH oxidase activity (Djordjevic et al. 2005). ET-1 induced NADPH oxidase derived O₂⁻ has been shown to phosphorylate ERK1/2 and that subsequently augments vascular SMC proliferation (Kyaw et al. 2002). A previous report indicated involvement of MMP-2 in vascular smooth muscle cell proliferation (Newby 2006). However, the role of MMP-2 in ET-1 mediated pulmonary artery smooth muscle cell proliferation and the underlying mechanism(s) is currently unknown. In a recent study, we observed that ET-1 induced increase in proMMP-2 expression and activation occurs via stimulation of NADPH oxidase activity (Sarkar et al. 2016). A pertinent question that may arise at this stage is whether proliferation of BPASMCs induced by ET-1 is linked with NADPH oxidase. The following pieces of evidence strongly support the involvement of NADPH oxidase derived O₂⁻ in MMP-2 mediated ET-1 induced PASMC proliferation. These are: (i) pretreatment of the cells with NADPH oxidase inhibitor, apocynin inhibited ET-1 induced increase in proliferation of the cells; (ii) pretreatment of the cells with apocynin inhibited ET-1 induced proMMP-2 expression and activation; and (iii) pretreatment of the cells with the MMP-2 specific inhibitor, MMP-2 inhibitor-1, attenuates ET-1 induced increase in the SMC proliferation.

Sphingomyelin (Spm) can be converted to ceramide (Cer) by neutral sphingomyelinase, which in turn forms sphingosine, and then produces sphingosine-1-phosphate (S1P) by the action of sphingosine kinase (SPHK). Sphingosine-Ceramide pathway has been shown to play a pivotal role in oxLDL induced SMC proliferation (Augé et al. 2004). S1P generated by SPHK is known to play an important role in pulmonary hypertension and subsequently promotes PASMC proliferation (Chen et al. 2014). Importantly, MMP-2 was shown to contribute to oxLDL induced activation of Spm-Cer signalling axis and that in turn produces the SMC proliferation (Augé et al. 2004). A previous research indicated that Spm-Cer-S1P pathway contributes to tissue plasminogen activator (tPA) induced mitogenic signalling pathways (Maupas-Schwalm et al. 2004).

In the present study we observed an increase in SMase activity and S1P level and proliferation of BPASMCs upon treatment with ET-1. The ET-1 induced responses were observed to be inhibited upon pretreatment of the cells with pharmacological and genetic inhibitors of SMase and SPHK. Taken together, these observations suggest that ET-1 induced proliferation of the SMCs occur via Spm-Cer-S1P signalling axis. Our present study also demonstrate that pretreatment of the SMCs with both pharmacological and genetic inhibitors of MMP-2 significantly attenuate ET-1 induced stimulation of SMase activity and S1P level and subsequently the smooth muscle cell proliferation. Thus, MMP-2 plays a crucial role in inducing the SMCs proliferation by activating the Spm-Cer-S1P signalling axis during ET-1 stimulation.

Fig. 11 Interaction of EGCG/EGC with NADPH oxidase p47phox subunit. **a** Comparison of interaction energies (Total IE), van der Waals energies (vdW), electrostatic energies (elec) and binding free energies (dG) by EGCG and EGC. **b** Residues that contributed interaction

energy < -4 kcal/mol. **c** 2D representation of p47phox-EGCG (red) interaction. **d** 2D representation of p47phox-EGC (green) interaction. **e** 3D ribbon representation of p47phox-EGCG (red) complex. **f** 3D ribbon representation of p47phox-EGC (green) complex

ET-1 treatment to the cells increases SIP level, which markedly, but not fully, returns to basal condition upon pre-treatment with the pharmacological and genetic inhibitors of SMase. However, ET-1 induced increase in SIP level has been

observed to be completely inhibited by both the pharmacological and genetic inhibitors of SPHK. This suggests that SIP production by ET-1 in the cells occurs via involvement of Spm-Cer-SIP signalling pathway. Notably, chemical and

genetic inhibitors of SMase are unable to inhibit ET-1 induced SPHK phosphorylation in the cells. However, these inhibitors significantly, but not fully, attenuate S1P production. This suggests that other component(s) also play important roles in the increase in S1P production during SPHK activation by ET-1 in the cells. Our present study also suggests that SPHK activation occurs via ERK1/2, which is evident by the following observations: (i) ET-1 treatment to the cells augments ERK1/2 phosphorylation; and (ii) the ERK1/2 inhibitor, PD98059 attenuates ET-1 induced increase in SPHK phosphorylation.

EGCG is known to ameliorate pulmonary hypertension (Zhu et al. 2017). EGCG has been shown to attenuate PDGF and FGF induced increase in vascular smooth muscle cell proliferation (Hwang et al. 2002; Sachinidis et al. 2002; Weber et al. 2004). In CL1-5 lung cancer cells, EGCG has been observed to attenuate SMC proliferation by suppressing MMP-2 expression and activation (Deng and Lin 2011).

Our study suggest that EGCG markedly attenuate ET-1 induced increase in MMP-2 activity, SMase activity, S1P level, SPHK and ERK1/2 phosphorylation and the SMC proliferation. The inhibitory effect of EGCG on ET-1 induced increase in the above parameters that leads to increase in SMCs proliferation appears to be due to the inhibition of NADPH oxidase activity. Our present study also suggests that EGCG inhibits ET-1 induced association of p47 phox with p67phox in the cell membrane. Inhibition of the association of p47phox

with p67phox by EGCG is also in keeping with our computational study. Our docking studies clearly indicate that galloyl group of EGCG is responsible for producing more than 30% higher binding free energy in occluding the PX domain of p47phox. The PX domain of p47phox is very much important as it has a role in translocation and protein-protein interaction. Presence of many charged residues at the binding site was clearly justified, producing more electrostatic interaction than the van der Waals or hydrophobic. Interestingly, due to lack of galloyl group, EGC is unable to show any distinct inhibitory effect towards p47phox, which is evident from minimum numbers of atomic contacts, H-bonds and π - π interactions. Thus, p47phox appears to be the target of EGCG, which by inhibiting association between p47phox and p67phox in the cell membrane leads to inhibition of ET-1 induced increase in NADPH oxidase derived $O_2^{\cdot -}$ production. EGCG appears to inhibit NADPH oxidase activity in a similar way to that of apocynin. This is based on a previous report that apocynin selectively hinder assembly of p47 with p67phox, components of NADPH oxidase complex, thereby inhibiting activation of NADPH oxidase (Beswick et al. 2001).

Thus, EGCG caused attenuation of ET-1 induced proliferation of BPASMCs appears to occur via inhibition of NADPH oxidase-MMP2-Spm-Cer-S1P signalling pathway. Figure 12 schematically represents the underlying mechanism of ET-1 induced proliferation of BPASMCs, and the role of EGCG in this scenario.

Fig. 12 Schematic representation of the role of EGCG in the ET-1 induced proliferation of the BPASMCs. Red marked portion of the structure of EGCG indicates the galloyl group

Conclusion

The present study suggests that the Spm-Cer-S1P pathway mediates ET-1 induced increase in proliferation of BPASMCs, which occurs via involvement of NADPH oxidase derived $O_2^{\cdot-}$ -mediated stimulation of MMP-2 activity. EGCG inhibits NADPH oxidase activity by preventing association between p47phox and p67phox (components of NADPH oxidase complex) in the cell membrane, thereby inhibiting MMP2-Spm-Cer-S1P signaling axis leading to inhibition of the SMCs proliferation. The molecular docking study suggests that p47phox is a potential target of the galloyl group of EGCG.

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Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interest.

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