Journal of Molecular and Cellular Cardiology 81 (2015) 23–33

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

PDE4 inhibition reduces neointima formation and inhibits VCAM-1 expression and histone methylation in an Epac-dependent manner

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ARTICLE INFO

Article history:
Received 31 August 2014
Received in revised form 21 January 2015
Accepted 22 January 2015
Available online 30 January 2015

Keywords:
Roflumilast
PDE4
Epac
VCAM-1
Epigenetic
Smooth muscle cells

ABSTRACT

Phosphodiesterase 4 (PDE4) activity mediates cAMP-dependent smooth muscle cell (SMC) activation following vascular injury. In this study we have investigated the effects of specific PDE4 inhibition with roflumilast on SMC proliferation and inflammatory activation in vitro and neointima formation following guide wire-induced injury of the femoral artery in mice in vivo. In vitro, roflumilast did not affect SMC proliferation, but diminished TNF-α induced expression of the vascular cell adhesion molecule 1 (VCAM-1). Specific activation of the cAMP effector Epac, but not PKA activation mimicked the effects of roflumilast on VCAM-1 expression. Consistently, the reduction of VCAM-1 expression was rescued following inhibition of Epac. TNF-α induced NFκB p65 translocation and VCAM-1 promoter activity were not altered by roflumilast in SMCs. However, roflumilast treatment and Epac activation repressed the induction of the activating epigenetic histone mark H3K4me2 at the VCAM-1 promoter, while PKA activation showed no effect. Furthermore, HDAC inhibition blocked the inhibitory effect of roflumilast on VCAM-1 expression. Both, roflumilast and Epac activation reduced monocyte adhesion to SMCs in vitro. Finally, roflumilast treatment attenuated femoral artery intima-media ratio by more than 50% after 4 weeks. In summary, PDE4 inhibition regulates VCAM-1 through a novel Epac-dependent mechanism, which involves regulatory epigenetic components and reduces neointima formation following vascular injury. PDE4 inhibition and Epac activation might represent novel approaches for the treatment of vascular diseases, including atherosclerosis and in-stent restenosis.

1. Introduction

Cyclic adenosine monophosphate (cAMP) regulates various cellular functions in virtually all cell types, making it one of the most important second messenger molecules in cellular signaling [1]. CAMP signaling is embedded into a highly sophisticated system of proteins governing its synthesis, degradation and cellular actions in a cell type and context specific manner. In this system degradation of cAMP is catalyzed by 3′,5′-cyclic nucleotide phosphodiesterases (PDEs) [2]. PDE-dependent regulation of cAMP-signaling is organized by compartment specific expression of PDEs, which results in the generation of intracellular cAMP gradients [3]. PDEs thereby exist in a large diversity of 11 distinct family members with numerous isoforms and splice variants being yet identified [4,5]. Of particular interest to the cardiovascular system are members of the PDE4 family. Vascular smooth muscle cells (SMCs) express PDE4A, PDE4B, as well as numerous variants of PDE4D [3]. While quiescent SMCs rely primarily on PDE3 for hydrolysis of cAMP, PDE4 activity surpasses PDE3 activity in SMCs that have been activated

Abbreviations: PDE4, Phosphodiesterase 4; SMC, Smooth muscle cell; CAM, Cell adhesion molecule; VCAM-1, Vascular cell adhesion molecule-1; ICAM-1, Intercellular adhesion molecule 1; PKA, Protein kinase A; Epac1, Exchange protein directly activated by cAMP; H3K4me2, Histone H3-lysine 4 dimethylation; HDAC, Histone deacetylase; cAMP, Cyclic adenosine monophosphate; BrdU, Bromodeoxyuridine; PDGF-BB, Platelet-derived growth factor BB; 6-MB-cAMP, N6-monobutyryladenosine-3′, 5′-cyclic monophosphate; 8CPT-2-O-Me-cAMP, 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2-O-Me-cAMP); NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; MCP-1, Monocyte chemotactic protein 1.

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http://dx.doi.org/10.1016/j.yjmcc.2015.01.015
0022-2828/

by growth factors or inflammatory cytokines [6,7]. SMCs that become activated at sites of vascular injury undergo a phenotypic switch, in which silencing of contractile genes is paralleled by an increased cellular propensity to migrate, proliferate and secrete extracellular matrix proteins [8]. These activated or synthetic SMCs constitute an essential component of pathologic vascular remodeling and atherosclerosis formation [8,9]. Abundant evidence has previously shown that increased cellular cAMP levels inhibit SMC activation [10–12]. Upregulation of PDE4 in response to inflammatory stimuli is consequently an obligate requirement for cAMP degradation and ensuing cell activation [13]. Considering the shift from PDE3 to PDE4 activity occurring during SMC phenotypic switch, PDE4 inhibition has been implicated as a promising target to selectively repress SMC driven remodeling following vascular injury.

Recently, roflumilast has been approved as the first selective PDE4 inhibitor for the treatment of chronic obstructive pulmonary disease (COPD) [2]. Roflumilast has been suggested to target several different pathogenetic mechanisms, including inflammation, fibrotic, emphysematous and vascular remodeling, as well as oxidative stress in COPD. The introduction of roflumilast into clinical practice has therefore provided a unique therapeutic opportunity to target PDE4 inhibition during various diseases mediated by inflammation and tissue remodeling [2,3]. In the present study, we have investigated the effect of selective PDE4 inhibition by roflumilast on SMC proliferation and inflammation as well as neointima formation in vivo.

2. Materials and methods

2.1. Cell culture

Rat aortic and human coronary SMCs were commercially obtained (Sigma Aldrich and Invitrogen, respectively). For all experiments cells were serum-deprived for 48 h. Cells were pretreated with roflumilast (Santa Cruz), the PKA activator N6-Monobutyryladenosine-3′, 5′-cyclic monophosphate (6-MB-cAMP) (BioLog), the Epac activator 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2-O-Me-cAMP) (BioLog), the Epac inhibitor 3-[5-(tert.-Butyl) isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitrile (ESI-09) (BioLog) or only dimethylsulfoxide (DMSO) used as solvent for all compounds for 30 min followed by stimulation with 10% FBS, 25 ng/mL platelet-derived growth factor BB (PDGF, Sigma Aldrich) or 10 ng/mL TNF-α (Sigma Aldrich), as indicated. For all data shown, cells were used between passages 3 and 7.

2.2. Cell proliferation assays

Cell proliferation was analyzed by cell counting using a hemocytometer and by BrdU incorporation (2750; Chemicon Millipore) according to the manufacturer's protocol. For cell cycle analysis, rat aortic SMCs were fixed in ice-cold 70% ethanol, incubated in PBS containing 40 μg/mL RNase for 30 min at 37 °C and resuspended in PBS containing 50 μg/mL propidium iodide. Cell cycle distribution was analyzed using a FACSCanto II Flow Cytometry System (Becton Dickinson).

2.3. Western blotting

The levels of CyclinD1 and VCAM-1 were determined by western blotting using specific antibodies as described previously [14]. Rat aortic SMCs were harvested in cell lysis buffer and whole cell proteins were subjected to immunoblotting using the following antibodies: CyclinD1 (2926; Cell Signaling), VCAM-1 (sc-8304; Santa Cruz) and β-Actin (A2228; Sigma-Aldrich).

2.4. RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction

RNA was isolated using the RNeasy Mini Kit (74106; QIAGEN) and reverse transcribed using SuperScript III (18080-051; Invitrogen) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction analysis of target gene expression was performed using a ViiA7 Cycler (Applied Biosystems) and Sybr GreenER qPCR Super Mix for ABI Prism (11760-500; Invitrogen). Each sample was normalized to mRNA expression of the housekeeping gene TFIIB. PCR primer sequences are available on request.

2.5. Apoptosis assay

Apoptosis was analyzed by measuring apoptosis 48 h after stimulation with roflumilast and 25 ng/mL PDGF using the Titer TACS In Situ Detection Kit (R&B) according to the manufacturer's protocol.

2.6. Rap1 assay

Serum-deprived rat aortic SMCs were treated with 1 μmol/L roflumilast or DMSO for 30 min and harvested in cell lysis buffer. Pull-down assays to quantify active Rap1 were performed with the Active Rap1 Pull-Down and Detection Kit (16120; Thermo Scientific) according to the manufacturer's instructions. Briefly, GST-RalGDS-RBD was added to spin cups containing glutathione resin and 500 μg cell lysates were immediately added. The reaction mixture was incubated at 4 °C for 1 h with gentle agitation. Precipitates were then washed, resuspended in 50 μL of 2× sample buffer, boiled for 5 min at 95 °C, and separated by 12% SDS-PAGE. Rap1 antibody was used to detect active Rap1. Whole-cell lysates were used to assess total levels of Rap1.

2.7. RNA interference

Human coronary SMCs were transfected with 50 nmol/L siRNA against Epac1 or non-targeting siRNA (Dharmacon) using RNAiMax transfection reagent (Invitrogen). Following transfection for 12 h, cells were serum deprived for 24 h and stimulated as indicated.

2.8. NFκB activation assay

NFκB activation was measured in rat aortic SMCs. Cells were serum deprived, pretreated with roflumilast or DMSO and stimulated with 10 ng/mL TNF-α for 6 h. NFκB p65 activation was measured using the TransAM ELISA kit (Active Motif) according to the manufacturer's instructions.

2.9. Luciferase reporter assay

Human coronary SMCs were transfected with a human 3.0 kb VCAM-1 luciferase reporter construct using Lipofectamine 2000 (Invitrogen) and treated with 10 ng/mL TNF-α, roflumilast or DMSO as described [15]. Transfection efficiency was normalized to renilla luciferase activities generated by cotransfection with pGL4.74[hRluc/TK] (Promega). Luciferase activity was assayed 24 h after transfection using a Dual Luciferase Reporter Assay System (Promega).

2.10. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed using the MAGnify ChIP-Kit (Invitrogen) according to the manufacturer's instructions. Briefly, serum-deprived human coronary SMCs were treated as described above and stimulated with 10% FBS. Cells were cross-linked in formaldehyde, harvested and soluble chromatin was prepared. Chromatin was immunoprecipitated using an antibody directed against histone H3 dimethylated at lysine 4 (Abcam) or control IgG (Invitrogen).