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Original article

ROS-dependent activation of RhoA/Rho-kinase in pulmonary artery: Role of Src-family kinases and ARHGEF1

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ABSTRACT

The role of reactive oxygen species (ROS) in smooth muscle contraction is poorly understood. We hypothesised that G-protein coupled receptor (GPCR) activation and hypoxia induce Rho-kinase activity and contraction in rat intra-pulmonary artery (IPA) via stimulation of ROS production and subsequent Src-family kinase (SrcFK) activation.

The T-type prostanoid receptor agonist U46619 induced ROS production in pulmonary artery smooth muscle cells (PASMC). U46619 also induced c-Src cysteine oxidation, SrcFK auto-phosphorylation, MYPT-1 and MLC_{20} phosphorylation and contraction in IPA, and all these responses were inhibited by antioxidants (ebselen, Tempol). Contraction and SrcFK/MYPT-1/MLC_{20} phosphorylations were also inhibited by combined superoxide dismutase and catalase, or by the SrcFK antagonist PP2, while contraction and MYPT-1/MLC_{20} phosphorylations were inhibited by the Rho guanine nucleotide exchange factor (RhoGEF) inhibitor Y16. H_2O_2 and the superoxide-generating quinoledione LY83583 both induced c-Src oxidation, SrcFK auto-phosphorylation and contraction in IPA. LY83583 and H_2O_2-induced contractions were inhibited by PP2, while LY83583-induced contraction was also inhibited by antioxidants and Y16. SrcFK auto-phosphorylation and MYPT-1/MLC_{20} phosphorylation was also induced by hypoxia in IPA and this was blocked by mitochondrial inhibitors rotenone and myxothiazol. In live PASMC, sub-cellular translocation of RhoA and the RhoGEF ARHGEF1 was triggered by both U46619 and LY83583 and this translocation was blocked by antioxidants and PP2. RhoA translocation was also inhibited by an ARHGEF1 siRNA. U46619 enhanced ROS-dependent co-immunoprecipitation of ARHGEF1 with c-Src.

Our results demonstrate a link between GPCR-induced cytosolic ROS or hypoxia-induced mitochondrial ROS and SrcFK activity, Rho-kinase activity and contraction. ROS and SrcFK activate RhoA via ARHGEF1.

1. Introduction

Reactive oxygen species (ROS), previously thought to occur solely as damaging bi-products of metabolism, are now recognised as bona fide second messengers in normal cellular function, being produced in response to multiple stimuli, including G-protein-coupled receptor (GPCR) agonists and hypoxia [1,2], presumably exerting their effects through reversible oxidation of specific target proteins, primarily on cysteine residues [3–5]. Normally, the production and degradation of ROS is tightly controlled by cytosolic or membrane-bound oxidoreductase enzymes, antioxidant enzymes and cellular redox buffers [6,7], but in cardiovascular disease this control is lost, resulting in oxidative stress [8]. GPCR induce vascular ROS production primarily via NADPH oxidase [1,7], while in intra-pulmonary arteries (IPA), hypoxia does so via the mitochondrial electron transport chain (complex III), an essential step in hypoxic pulmonary vasoconstriction [2,9,10]. Most contractile stimuli, including GPCR and hypoxia, exert their effects on smooth muscle cross-bridge cycling via a combination of raised $[Ca^{2+}]_i$ and inhibition of myosin light-chain phosphatase [11–13], the latter being dependent on activation of Rho-kinase [14]. Applied exogenously, both superoxide ($O_2^{\cdot-}$) and its dismutation product H_2O_2 have contractile effects in pulmonary artery, but previous work from our laboratory suggests that exogenous H_2O_2 *primarily* does so via activation of PKC and elevation of $[Ca^{2+}]_i$, while artificially induced cytosolic superoxide *primarily* activates Rho-kinase [15,16].

The role of endogenous ROS in Rho-kinase activation by GPCR or hypoxia remains to be fully characterised because the signalling pathway(s) through which ROS may activate Rho-kinase are unclear.

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Regardless of the initial stimulus, Rho-kinase activity usually requires prior activation of monomeric G-proteins of the Rho family (including RhoA), which in turn normally requires prior activation of guanine nucleotide exchange factors selective for the Rho family of G-proteins (RhoGEFs) [17]. Thus, either RhoA itself is directly ROS-sensitive [18] or RhoGEF activation may be triggered by ROS independently of the canonical $G_{12}/_{13}$ interaction [19,20].

Increased Rho-kinase activity contributes to acute hypoxic pulmonary vasoconstriction [11,12], as well as elevated pulmonary artery pressure in chronic hypoxia-induced pulmonary hypertension [21], but how this increased activity is mediated remains unclear. Non-receptor tyrosine kinases contribute to aberrant migratory and proliferative responses in oxidative stress-induced vascular remodelling [22,23], but are also involved in normal contractile function in vascular smooth muscle. For example, we showed that in IPA both GPCR-induced and hypoxia-induced Rho-kinase activity and contraction was dependent on prior activation of Src-family kinases (SrcFK) [11,24]. In non-muscle cells, c-Src has been shown to be directly ROS-sensitive, with cysteine oxidation enhancing its activity [25], though it may also be activated indirectly through oxidative inhibition of its negative regulators, such as c-Src kinase or specific tyrosine phosphatases (reviewed in [4,25]). It has not been determined whether SrcFK are ROS-sensitive in IPA, nor whether ROS- and/or SrcFK-mediated activation of RhoA/Rho-kinase occurs as part of the same signalling pathway in response to GPCR or hypoxia, but it is possible that SrcFK act as intermediaries between ROS and RhoA/Rho-kinase. We therefore hypothesised that SrcFK act as key mediators of ROS signalling in IPA, contributing to GPCR and hypoxia-induced RhoA/Rho-kinase activity and contraction. We also examined the role of ARHGEF1, an RGS-domain containing RhoGEF that has previously been shown to be activated by tyrosine phosphorylation [26].

2. Methods

2.1. Tissue and cell culture

This study conforms with UK Home Office regulations and Directive 2010/63/EU of the European Parliament. Adult male Wistar rats were killed by lethal overdose of pentobarbital (~50 mg/kg i.p.). The lungs were excised and placed in cold physiological saline solution (PSS, composition in mM: 118NaCl, 24 $NaHCO_3$, 1 $MgSO_4$, 4 KCL, 5.56 D-glucose, 0.434 NaH_2PO_4, 1.8 $CaCl_2$, pH 7.4). Small intra-pulmonary arteries (IPA; 200–500 μm i.d.) were dissected free of surrounding parenchyma and either used for protein extraction and immunoblotting, mounted on a myograph for measurement of contractile force, or used for preparation of cultured pulmonary artery smooth muscle cells (PASMC). PASMC were dispersed by enzymatic digestion (collagenase type XI, papain, trypsin inhibitor), grown to passage 3–4 in DMEM and serum-starved for 24 h prior to use. Each batch of cells was verified as smooth muscle by immunostaining for smooth muscle α-actin, calponin and desmin, as shown previously [1].

2.2. Contractile force measurement

IPA rings were mounted on a Mulvany-Halpern wire myograph (DMT.dk) bathed in PSS and gassed with 95% air/5% CO_2 (pH 7.4) at 37 °C. Vessels were stretched and pre-conditioned by stimulation with repeated exposures to 80 mmol/l K^+ PSS (KPSS, equimolar substitution for NaCl), with resting tone being set at 1–3 mN, as previously described [24]. Experiments were performed after ~30 min to allow for stabilization. Tension was recorded using Acquisition Engine software (Cairn Research Ltd, Faversham, UK).

2.3. Immuno-precipitation (IP) and Western blot (WB)

Following isolation and treatment with U46619 under normoxic conditions (5% CO_2/95% air) or hypoxic conditions (1% O_2/5% CO_2, 94% N_2), IPA tissue samples were snap frozen in liquid nitrogen, homogenised and protein extracted for IP and/or SDS-PAGE and Western blot. For IP followed by WB, protein was extracted in TRIS-buffered saline with triton (1%), whereas for immediate WB, protein was extracted in TRIS-buffered saline with SDS (5%). IP was performed using the Pierce™ Crosslink Magnetic IP/Co-IP Kit (8805) according to the manufacturer instructions. Briefly, lysates were incubated with anti-c-Src antibody (1:20, Cell Signalling) and then covalently bound to protein A/G Magnetic Beads (Pierce) overnight at 4 °C. Following appropriate washing steps and elution, immuno-precipitates were re-suspended in TRIS-SDS sample buffer.

For SDS-PAGE, gel total protein loading was normalised by BCA assay. Membranes were blocked for 1hr at room temperature with 5% skimmed milk in TBS-buffered saline with 0.05% Tween (TBS-T) then probed with primary antibodies overnight at 4 °C (in 5% milk/TBS-T). Dilutions were optimised for each primary antibody, typically 1:1000, followed by horseradish peroxidase-conjugated secondary antibodies for 1hr at room temperature (1:3000 in 5% milk/TBS-T). Membranes were first probed with anti-phospho-antibodies, then stripped for 1hr (Pierce stripping buffer), re-blocked and re-probed with anti-total antibodies. Protein bands were visualised using either SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) or ECL Prime (Amersham, GE heatlthcare) in a Biorad ChemiDoc XRS+ Gel Imaging System. Band intensity was expressed as a ratio of phospho/total for each protein band of interest, and for each treated sample these ratios were then expressed as a percentage of the control (untreated) samples run on the same gel. Each control value was taken as the average of 2–3 identically treated samples on each gel.

2.4. ROS measurement

PASMC were seeded into 96-well plates, grown to 80–90% confluence and serum-starved for 24hrs. Cell were then incubated with the luminol-derived superoxide probe 8-amino-5-chloro-7-phenylpyrido [3,4-*d*]pyridazine-1,4-(2 H,3 H)-dione (L-012, 50 μM, Wako Pure Chemical Industries) [27], in PBS at 37 °C. We also used an alternative chemi-luminescence ROS probe (ROS-Glo™, Promega) [28] to measure H_2O_2 production in IPA. Changes in luminescence induced by acute drug treatments were detected using a Promega GloMax Multi+ plate reader. Background measurements using ROS probe plus drug, but in the absence of cells/tissue, were subtracted from all equivalent test readings in the presence of cells/tissue. Each final value for each treatment for each batch of cells was the average of measurements from at least 8 identically treated wells in each plate.

2.5. c-Src oxidation assay

Reversible oxidation of c-Src cysteine residues in response to acute treatment with U46619 or exogenous ROS was determined using the semi-quantitative PEG-switch method [29]. Briefly, this involves incubating IPA lysates with maleimide (100 mM, 25 min, 50 °C) to alkylate reduced cysteine residues. Then, following reduction of reversibly oxidised cysteines with dithiothreitol (DTT, 200 mM, 30 min, RT°C) and desalting to remove maleimide and DTT (Zeba Spin desalting columns, Thermo Scientific), lysates are incubated with polyethyleneglycol 5000-tagged maleimide (PEG-maleimide, 10 mM, 2 h, RT°C), to alkylate remaining reduced cysteine residues. When subjecting the lysate to SDS-PAGE and probing for c-Src by Western blot, reversible oxidation is indicated by separation of c-Src into two or more bands due to oxidised cysteine pegylation increasing the protein molecular weight by multiples of 5KDa.

2.6. cDNA cloning, siRNA design and cell transfection

Rat RhoA cDNA was cloned into PCR2.1-TOPO® TA vector