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AMP-activated protein kinase and hypoxic pulmonary vasoconstriction

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ABSTRACT

Hypoxic pulmonary vasoconstriction is a vital homeostatic mechanism that aids ventilation–perfusion matching in the lung, for which the underlying mechanism(s) remains controversial. However, our most recent investigations strongly suggest that hypoxic pulmonary vasoconstriction is precipitated, at least in part, by the inhibition of mitochondrial oxidative phosphorylation by hypoxia, an increase in the AMP/ATP ratio and consequent activation of AMP-activated protein kinase (AMPK). Unfortunately, these studies lacked the definitive proof that can only be provided by selectively blocking AMPK-dependent signalling cascades. The aim of the present study was, therefore, to determine the effects of the AMPK inhibitor compound C upon: (1) phosphorylation in response to hypoxia of a classical AMPK substrate, acetyl CoA carboxylase, in rat pulmonary arterial smooth muscle and (2) hypoxic pulmonary vasoconstriction in rat isolated intrapulmonary arteries. Acetyl CoA carboxylase phosphorylation was increased approximately 3 fold in the presence of hypoxia (pO_2 = 16–21 mm Hg, 1 h) and 5-aminoimidazole-4-carboxamide riboside (AICAR; 1 mM; 4 h) and in a manner that was significantly attenuated by the AMPK antagonist compound C (40 μM). Most importantly, pre-incubation of intrapulmonary arteries with compound C (40 μM) inhibited phase II, but not phase I, of hypoxic pulmonary vasoconstriction. Likewise, compound C (40 μM) inhibited constriction by AICAR (1 mM). The results of the present study are consistent with the activation of AMPK being a key event in the initiation of the contractile response of pulmonary arteries to acute hypoxia.

1. Introduction

Hypoxic pulmonary vasoconstriction is the critical and distinguishing characteristic of the arteries within the lung (von Euler and Liljestrand, 1946) and contributes to ventilation–perfusion matching by diverting blood flow to O_2-rich areas of the lung. The precise mechanism(s) by which hypoxia elicits hypoxic pulmonary vasoconstriction is a contentious issue, although it is clear that relatively mild hypoxia inhibits mitochondrial oxidative phosphorylation in pulmonary arterial smooth muscle (Archer et al., 1986; Duchen and Biscoe, 1992a,b; Leach et al., 2001; Shigemori et al., 1996; Youngson et al., 1993) and over a range of pO_2 that elicits no such response in cells that do not function to monitor O_2 supply (Duchen and Biscoe, 1992b). Despite this fact, however, little consideration has been given to the possibility that hypoxic pulmonary vasoconstriction may be dependent on the subsequent recruitment of AMP-activated protein kinase (AMPK) a central component of a highly conserved protein kinase signalling cascade that monitors the AMP/ATP ratio as an index of metabolic stress (Corton et al., 1995b; Hardie and Hawley, 2001; Marsin et al., 2000; Sakamoto et al., 2005; Shaw et al., 2005; Winder and Hardie, 1996). AMPK is a heterotrimer comprising a catalytic α subunit and regulatory β and γ subunits. It is activated by the binding of AMP to two sites on the γ subunit (Hawley et al., 1995; Scott et al., 2002) leading to allosteric activation of AMPK, phosphorylation of the α subunit at Thr-172 by an upstream kinase, for example the tumour suppressor kinase LKB1 (Hawley et al., 2003; Shaw et al., 2004, 2005; Woods et al., 2003) and the inhibition of AMPK dephosphorylation. These processes are normally antagonized by high concentrations of ATP and thereby provide a triple mechanism of regulation that is exquisitely sensitive to very small changes in the AMP/ATP ratio (Hardie and Hawley, 2001). It is generally accepted that AMPK functions to maintain ATP supply in all eukaryotic cells by activating catabolic processes and by inhibiting non-essential ATP consuming processes. However, AMPK is a serine threonine kinase and may regulate processes outside of metabolism (Hardie, 2005).

Our most recent investigations suggest that inhibition of mitochondrial oxidative phosphorylation by hypoxia does indeed increase

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the AMP/ATP ratio in pulmonary arterial smooth muscle, leading to consequent activation of AMPK and, in turn, hypoxic pulmonary vasoconstriction (Evans, 2006; Evans et al., 2006a,b, 2005). However, while these studies provided strong correlations between the effects of AMPK activation on pulmonary artery function and hypoxic pulmonary vasoconstriction, they fell short of demonstrating a causal role for AMPK in the latter process. Here, therefore, we have investigated the effects of the AMPK antagonist compound C on hypoxic pulmonary vasoconstriction.

2. Materials and methods

2.1. Pulmonary artery isolation and small vessel myography

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (250–350 g) were killed by decapitation and the heart and lungs rapidly excised and placed in a cold physiological salt solution (PSS) containing (in mM): 118 NaCl, 4 KCl, 1 $MgSO_4$, 0.435 NaH_2PO_4, 24 $NaHCO_3$, 1.8 $CaCl_2$, 5 Na-pyruvate, 5.6 glucose, pH 7.4, 37 °C. Small 3rd and 4th order branches of the pulmonary arterial tree (200–500 μM internal diameter, i.d.) were dissected free of connective tissue and mounted in a small vessel myograph (Model 500A, Danish Myo Technology, Denmark) as previously described in detail (Robertson et al., 2001) and gassed with 95% air/5% CO_2. Pulmonary artery rings were then equilibrated with 3 exposures to 80 mM K^+-PSS (KPSS, 2 min duration, isotonic replacement of Na^+ by K^+), as described previously (Robertson et al., 2001). To facilitate the hypoxic response, pulmonary arteries were exposed to 1 μM prostaglandin $F_2\alpha$ ($PGF_2\alpha$) for 30 min prior to, and during, the hypoxic challenge as previously described (Robertson et al., 2000). Hypoxia (16–21 mm Hg) was then induced by gassing with 1% O_2/95% N_2/5% CO_2 for 45 min, after which time the vessels were reoxygenated for 20 min, washed with PSS and subsequently re-exposed to KPSS. Compound C (10–40 μM, a gift from Merck Research Laboratories, Rahway, NJ) was added to the bathing solution 15 min prior to the induction of hypoxia. Time-matched controls were employed in all experimental protocols. The concentrations of compound C used were selected based upon previous reports where compound C had been found to inhibit AMPK (Lee et al., 2003; Zhou et al., 2001). Likewise, we studied the effect of compound C (40 μM) on constriction induced in response to (1) AMPK activation by 5-aminoimidazole-4-carboxamide riboside (AICAR; 1 mM) and (2) activation of the sarcoplasmic reticulum store-refilling current by the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (1 μM; without and then with extracellular Ca^{2+}; Mathes and Thompson, 1995).

2.2. Acetyl CoA carboxylase phosphorylation

All experiments were performed in accordance with the *United Kingdon Animals (Scientific Procedures) Act 1986*. For each sample 32 pulmonary arteries (2nd and 3rd order branches combined) were excised from 8 animals and carefully threaded onto silk surgical thread. They were then placed into an enclosed experimental chamber which was filled with PSS-B: 118 NaCl, 4 KCl, 1 $MgSO_4$, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 5.6 glucose, pH 7.4, 37 °C, and bubbled with 75% N_2, 20% O_2, 5% CO_2 (normoxia: 150–160 mm Hg) or 93% N_2, 2% O_2, 5% CO_2 (hypoxia: 16–21 mm Hg). Artery samples were exposed, in the absence and presence of compound C (40 μM), to either: (1) 2 h of normoxia, (2) 1 h of normoxia and 1 h of hypoxia or (3) 1 h of normoxia and 4 h in the presence of AICAR (1 mM). Tissue samples were then quickly removed from the chamber, placed in 1 ml centrifuge tubes and snap frozen in liquid nitrogen. Acetyl CoA carboxylase (ACC) phosphorylation and total ACC protein levels were analyzed using pre-cast 3–8% Tris-acetate gels in Tris-acetate buffer. ACC phosphorylation and total ACC protein were measured via dual labelling using phospho-specific antibodies against Ser-221 on ACC2, with secondary anti-sheep antibodies conjugated to IR680 (1 mg ml^{-1}), and streptavidin conjugated to IR800. Fluorescence from the two dyes was measured simultaneously using an Odyssey Infrared Imaging System (Li-Cor Biosciences) (Scott et al., 2002). All tissue samples were assayed in parallel.

2.3. Data presentation and statistical analysis

Contractile responses were calculated as a percentage of the maximal contractile response to KPSS ($\%T_K$) for each vessel. Data for small vessel myography are presented as mean ± S.E.M., while those for ACC phosphorylation represent the mean ± S.D. Data were analyzed by repeated measures analysis of variance (ANOVA). Differences between individual means were determined by Student's modified *t*-test using the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVA. A value of $P<0.05$ was deemed to be significant.

2.4. Drugs and chemicals

5-Aminoimidazole-4-carboxamide riboside (AICAR) and compound C were obtained from Calbiochem. All other drugs and chemicals were obtained from Sigma.

3. Results

3.1. Compound C inhibits ACC phosphorylation in response to hypoxia and AICAR in isolated pulmonary arteries

We first assessed the ability of compound C to inhibit ACC phosphorylation in response to hypoxia and to AMPK activation by AICAR, which is taken up into cells via the adenosine transporter and metabolised to form the AMP mimetic ZMP (Corton et al., 1995a; Owen et al., 2000), in pulmonary arterial smooth muscle. Under normoxia (150–160 mm Hg) the ratio of phosphorylated ACC/total ACC (pACC/ACC) in 2nd and 3rd order branches of the pulmonary arterial tree, without endothelium, measured 1.00 ± 0.04 in the absence and 0.58 ± 0.10 (mean ± S.D., $P<0.05$, $n=3$) in the presence of 40 μM compound C. Upon exposure to AICAR (1 mM) the pACC/ACC ratio rose to 4.23 ± 0.85 and in a manner that was reversed to 1.49 ± 0.47 ($P<0.05$; $n=3$) in the presence of 40 μM compound C. Likewise, hypoxia (16–21 mm Hg) increased the pACC/ACC ratio to 4.12 ± 1.03 in the absence and 2.82 ± 0.63 ($P<0.05$; $n=3$) in the presence of 40 μM compound C. Thus, compound C reduced the resting pACC/ACC ratio and inhibited the increase thereof in response to hypoxia and AMPK

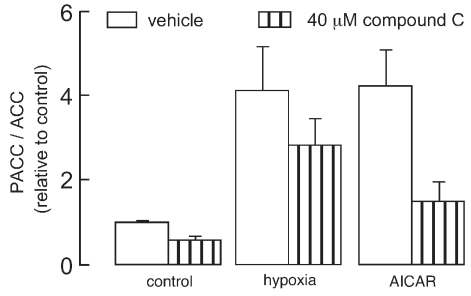

Fig. 1. Phosphorylation of acetyl CoA carboxylase in response to hypoxia and AICAR is inhibited by the AMPK antagonist compound C. Bar chart shows the phosphorylated acetyl CoA carboxylase/acetyl CoA carboxylase (PACC/ACC) ratio measured in pulmonary artery smooth muscle lysates under control conditions (2 h normoxia, 150–160 mm Hg), hypoxia (1 h at 16–21 mm Hg; following 1 h normoxia) and in the presence of 1 mM AICAR (4 h; following 1 h normoxia) with and without 40 μM compound C.