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Polyunsaturated fatty acid biosynthesis is involved in phenylephrine-mediated calcium release in vascular smooth muscle cells

Nicola A. Irvine [a], Karen A. Lillycrop [b], Barbara Fielding [c], Christopher Torrens [a], Mark A. Hanson [a], Graham C. Burdge [a,*]

[a] Academic Unit of Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK
[b] Centre for Biological Sciences, Faculty of Natural and Environmental Sciences, University of Southampton, Southampton, UK
[c] Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

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ABSTRACT

Stimulation of vascular smooth muscle (VSM) α_1-adrenoceptors induces myosin phosphorylation and vasoconstriction via mobilisation of intracellular calcium and production of specific eicosanoids. Polyunsaturated fatty acid (PUFA) biosynthesis in VSM cells is involved, although the precise mechanism is not known. To address this, we characterised PUFA biosynthesis in VSM cells and determined its role in intracellular calcium release and eicosanoid production. Murine VSM cells converted 18:2n-6 to longer chain PUFA including 22:5n-6. Δ6 (D6d) and Δ5 (D5d) desaturase, and elongase (Elovl) 5 were expressed. Elovl2 was not detected in human, mouse or rat VSM cells, or in rat or mouse aortae, but tit was not associated with hypermethylation of its promoter. D6d or D5d inhibition reduced 18:3n-6 and 20:4n-6 synthesis, respectively, and induced concentration-related decrease in phenylephrine-mediated calcium release, and in PGE_2 and $PGF_{2\alpha}$ secretion. Together these findings suggest that PUFA biosynthesis in VSM cells is involved in calcium release associated with vasoconstriction.

1. Introduction

Mammals can convert linoleic acid (18:2n-6) or α-linolenic acid (18:3n-3) to longer chain polyunsaturated fatty acids (PUFA) via a series of sequential desaturation and carbon chain elongation reactions [1,2]. The initial, rate limiting reaction which is catalysed by Δ6-desaturase (D6d) introduces a double bond at the Δ6 position. Two carbons are then added by the action of elongase-5. Further desaturation at the Δ5 position which is catalysed by Δ5-desaturase (D5d) desaturase generates 20 carbon PUFA; arachidonic acid (20:4n-6) or eicosapentaenoic acid (20:5n-3). These 20 carbon fatty acids can be elongated to 22 carbon intermediates by elongase-5 and then to 24 carbon PUFA by elongase2 or 5 activity. An additional double bond is then inserted at the Δ6 position by D6d and the resulting PUFA are then translocated from the endoplasmic reticulum to peroxisomes where the carbon chain is reduced by 2 carbons by one cycle of fatty acid β-oxidation [1].

Components of the PUFA biosynthesis pathway, D6d, D5d, elongase2 and 5 have been detected in a number of tissues [3,4]. D6d, D5d, elongase2 and 5 transcripts are highly expressed in liver but also present at lower levels in and brain, but not detected in heart [4]. D6d has also been detected in placenta, lung, skeletal muscle, kidney and pancreas [3,5]. D5d is expressed at markedly lower levels than D6d in these tissues. Synthesis of 20:4n-6 and 22:6n-3 has been reported in vascular endothelial [6,7] and smooth muscle cells [8], synthesis of dihomo-γ-linolenic acid (20:3n-6) has been reported in epidermis [9] and in macrophages [10], while 22:6n-3 synthesis has been detected in astrocytes [11]. The primary role of PUFA biosynthesis is generally assumed to support the synthesis of cell membranes. In the liver PUFA biosynthesis also provides substrates for the synthesis of lipoproteins which serve to supply PUFA to peripheral tissues. However, we have shown previously that PUFA biosynthesis in vascular smooth muscle (VSM) cells is involved in α_1-adrenoceptor (α_1AR)-mediated vasoconstriction [12].

Phosphorylation of the myosin light chain is the principal determinant of force development in vascular smooth muscle

* Correspondence to: Academic Unit for Human Development and Health, Faculty of Medicine, University of Southampton, IDS Building, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK.
Tel.: +44 2381205259; fax +44 2381204221.
E-mail address: g.c.burdge@soton.ac.uk (G.C. Burdge).

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(VSM) [13]. This is initiated by the release of calcium from intracellular stores in response to the activation of G protein-coupled receptors by neuroendocrine factors, for example binding of phenylephrine (Pe) to α_1AR. This leads to activation of protein kinase C that, via various second messengers including eicosanoids, facilitating efflux of calcium into the cytoplasm, producing calcium sensitisation [13]. Increased myosin phosphorylation also involves inhibition of myosin light chain phosphatase (MLCP) [14], hence preventing dephosphorylation of myosin, via the activities of atypical protein kinase C (aPKCζ) [15,16] and rhoA kinase [17,18]. Non-esterified 20:4n-6 has been shown to inhibit MLCP leading to increased myosin light chain phosphorylation and impaired vasodilatation in VSM independent of intracellular calcium concentration and the production of eicosanoid synthesis [19]. However, this effect was inhibited by non-enzymatic oxidation of 20:4n-6 indicating that intact 20:4n-6, and not its metabolites, was responsible for inhibition of MLCP [19]. A calcium independent phospholipase A2 has also been implicated in calcium sensitisation in VSM cells [20].

We have shown previously that SC26196, a specific inhibitor of D6d [8,21], or sesamin, a specific inhibitor of D5d [22], reduced Pe-mediated vasoconstriction in rat aortae and human femoral arteries *ex-vivo* by approximately 75% irrespective of the presence or absence of the vascular endothelium [12]. These findings suggest that, in addition to any role in maintaining membrane fatty acid composition, PUFA biosynthesis in VSM cells is linked closely to the process of α_1AR-mediated force generation. Treatment of isolated rat aortae with SC26196 reduced selectively the secretion of the pro-vasoconstriction eicosanoids prostaglandin (PG)$F_{2\alpha}$, PGE_2 and thromboxane (Tbx) B2 [12]. Since eicosanoids acting via thromboxane receptors in VSM are potent agonists for vasoconstriction [23], these findings suggest that one possible role for PUFA biosynthesis in developing VSM tone could be to generate a source of substrates for the formation of vasoactive prostanoids. However, it is not known whether PUFA biosynthesis in VSM cells is induced by Pe, which would suggest coordinated regulation of PUFA formation with dynamic changes in blood flow, or whether it is constitutive and hence possibly involved in vascular homoeostasis. Furthermore, it is not known whether PUFA biosynthesis is in involved directly in Pe-mediated calcium release or downstream of this event, for example by providing substrates that influence myosin kinase or phosphatase activities. To address this, we characterised PUFA biosynthesis in VSM cells *in vitro* and measured the effect of Pe on the activity of the PUFA biosynthesis pathway. We also determined the effect of inhibiting PUFA biosynthesis on Pe-induced release of intracellular calcium and production of pro-vasoconstriction eicosanoids.

2. Materials and methods

2.1. Materials

SV40-immortalised murine vascular smooth muscle (MOVAS) cells were from American Type Culture Collection (Manassas, Virginia, USA), murine hepatoma cells (Hepa1-6) and human hepatocarcinoma cells HepG2 were from European Collection of Cell Cultures (Porton Down.

Salisbury, UK) and human primary aortic smooth muscle (HASM) cells were from Life Technologies. [1-^{13}C]-18:2n-6 ethyl ester was purchased from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA). Primers for real time PCR were from Qiagen (Manchester, UK). Bespoke pyrosequencing PCR and sequencing primers were purchased from Biomers (Ulm, Germany). All other reagents were obtained from Sigma or PAA, with noted exceptions. Mouse and rat aorta and liver tissue that were used to confirm findings in immortalised cells were archived specimens collected from adult animals that had been maintained throughout life on RM1 standard chow diet (Special Diets Services, Witham, Essex, UK) and which were stored at −80 °C.

2.2. Measurement of PUFA biosynthesis

n-6 PUFA biosynthesis was measured directly by the incorporation of [^{13}C] from 18:2n-6 into longer chain metabolites or indirectly from the proportions of individual n-6 PUFA in total cell lipid extracts following treatment with linoleic acid.

In order to measure incorporation of [^{13}C] into n-6 PUFA, HEPa1-6 or MOVAS cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (10% (w/w)) at sub-confluency and allowed to attach for 24 h. [1-^{13}C]18:2n-6 ethyl ester (10 μmoles/l) and 18:2n-6 (90 μmoles/l, vehicle) were added and the cultures which were maintained at 37 °C in an atmosphere containing 5% (v/v) CO_2 for 48 h. In some experiments, Pe (100 μmol/l) was added to the MOVAS cell cultures for the last 30 min. This Pe concentration has been shown to induce vasoconstriction in *ex-vivo* preparations of rat and human arteries [12]. At the end of the incubation period, cells were washed with ice cold Hank's Balanced Salt Solution (HBSS). 0.8 ml NaCl (0.9% w/v) was then added and the cells scraped into a glass tube and placed on ice. An aliquot was reserved for measurement of total cell protein using the Pierce® BCA Protein Assay kit (Thermo Scientific).

Incubation of cells with unlabelled 18:2n-6 were carried out in an identical manner to those which used [^{13}C]18:2n-6 except 18:2n-6 was added to a final concentration of 100 μmol/l.

2.3. Measurement of stable isotope incorporation by gas chromatography combustion isotope-ratio mass spectrometry

Total lipids were extracted with chloroform and methanol [24] using heptadecanoic acid (10 μg) as internal standard. Fatty acids were converted to fatty acid methyl esters (FAMEs) by incubation with methanol containing 2% (v/v) sulphuric acid [25]. The concentration of individual PUFA was determined by gas chromatography [10]. Incorporation of [^{13}C] into n-6 PUFA was measured by gas chromatography combustion isotope-ratio mass spectrometry as described [26]. Briefly, FAMES were separated on a gas chromatograph (Thermo trace GC ultra, Bremen, Germany) equipped with a DB-wax column (30 m × 0.25 mm × 0.25 μm) (Agilent, UK). FAMES were then oxidatively combusted to CO_2, which was ionised and detected using a Thermo Finnigan Delta^{Plus} XP mass spectrometer allowing the ratio of $^{13}CO_2/^{12}CO_2$ from the combusted product to be measured, calibrated against a certified standard (eicosanoic acid FAME, Department of Geological Sciences, Indiana University, Bloomington, IN). FAMES were identified from retention times of standards (Sulpelco 37 component FAME mix and individual FAME standards, Sigma-Aldrich, Dorset, UK).

2.4. Measurement of fatty acid composition by gas chromatography

Total lipids and FAMEs were prepared as above. The proportions of individual fatty acids were measured by gas chromatography using an Aglient 6890 gas chromatograph equipped with a BPX70 fused silica capillary column (30 m × 0.25 mm × 0.25 μm) (SGE) and flame ionisation detection [12]. FAMEs were identified by their retention times relative to standards and quantified using Chemstation software (Agilent).

2.5. Measurement of mRNA expression

Total RNA was isolated from cells using Tri-reagent (Sigma) as described [12]. Complementary DNA was prepared and amplified using real-time RTPCR, which was performed as described using