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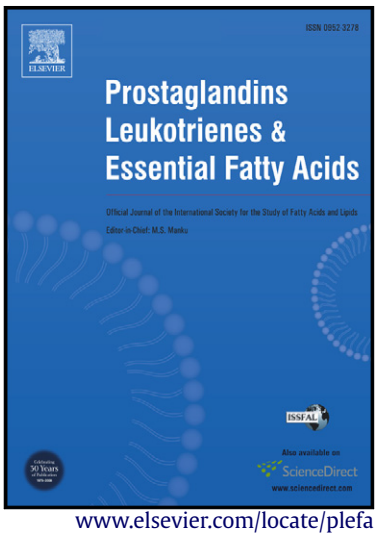

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Polyunsaturated fatty acid biosynthesis is required for phenylephrine-mediated calcium release in vascular smooth muscle cells

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.

*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.: +442381205259; fax +442381204221. E-mail address: g.c.burdge@soton.ac.uk

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

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human, mouse or rat VSM cells, or in rat or mouse aortae, but twas 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 calcium release associated with vasoconstriction.

Keywords

Polyunsaturated fatty acids; vascular smooth muscle cells; calcium

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 catalyzed 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 elongase 2 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, elongase 2 and 5 have been detected in a number of tissues [3,4]. D6d, D5d, elongase 2 and 5 transcripts are highly expressed in liver but also present at lower levels in and brain,

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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 (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-

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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) B_2 [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 homeostasis. 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.

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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 immortalized 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^{o}C.

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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 hours. [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^{o}C in an atmosphere containing 5% (v/v) CO_2 for 48 hours. In some experiments, Pe (100 µmol/l) was added to the MOVAS cell cultures for the last 30 minutes. 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). 0.8ml 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 x 0.25 mm x 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 $^{13}CO_2/^{12}CO_2$

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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 (30m x 0.25 mm x 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).

2.3 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 SYBR Green Jumpstart Ready Mix (Sigma) in a final volume of 25 µl [12]. RTPCR primers are listed in Table 1. All samples were analysed in duplicate. Cycle parameters were 95^{o}C for 2 minutes then 40 cycles of 95^{o}C for 30 seconds, 55^{o}C (cyclophilin, FADS1, FADS2, ELOVL2 and ELOVL5) for 1 minute and 72^{o}C for 1 minute. Expression was determined by the standard curve method [27] using cyclophilin (Qiagen, West Sussex, UK) as the reference gene.

2.4 Measurement of the DNA methylation status of the ELOVL2 promoter

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Genomic DNA was extracted from VSM and liver cells, and mouse aortae and liver samples as described [28]. Briefly, DNA was treated with sodium metabisulphite using the EZ DNA methylation kit (ZymoResearch). The pyrosequencing reaction was carried out using primers listed in Table 2. Modified DNA was amplified using KAPA2G Robust Hot Start Taq DNA polymerase (Labtech). The amplified products were immobilised on streptavidin–sepharose beads (GE Healthcare UK Ltd.), washed, denatured and released into annealing buffer containing the sequencing primers (Table 2) (Biomers, Söflinger, Germany). Pyrosequencing was carried out using an SQA kit on a PSQ 96MA pyrosequencer (Biotage, Uppsala, Sweden). The percentage methylation at each CpG locus was determined using the Pyro Q CpG software (Biotage).

2.5 Measurement of calcium release

MOVAS cells were seeded in DMEM at approximately 40,000 per well in a black-sided, clear base 96 well plate (Greiner Bio-One Ltd) and allowed to attach for 24 hours. The Screen Quest Fluo-8 Medium kit was prepared according to the manufacturer's instructions (AAT Bioquest). Fluo-8 dye was dissolved in dimethylsulphoxide (DMSO) and then mixed with HBSS and 10x pluronic F127. The cell culture medium was replaced with dye solution and either SC26196 or sesamin dissolved in HBSS containing DMSO (0.1% v/v) or DMSO vehicle solution alone (0.1% v/v) were then added. Cells were incubated in the dark for 30 minutes at 37^{o}C and then for a further 30 minutes at room temperature. In some experiments, Pe (10 μmol/l) was added for 30 seconds prior to reading the plate using a Thermo Labsystems Fluoroscan at an excitation wavelength λ = 485 nm and emission

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wavelength λ = 538 nm. A23187 calcium ionophore (500 nmoles/l) was used as the positive control.

2.6 Measurement of eicosanoid concentrations

MOVAS cells were seeded at approximately 60,000 cells per well in 6 well plates in DMEM and allowed to attach as before. Either SC26196 (200 nmol/l to 1 µmol/l) or sesamin (10 µmol/l to 20 µmol/l) was added and the cells incubated for a further 48 hours. The medium was then replaced with DMEM without fetal bovine serum containing either SC26196 or sesamin, with or without Pe (100 µmol/l). Cells were maintained for a further 30 minutes. The culture supernatant was collected, frozen immediately on dry ice and the samples stored at -80^{o}C. The concentrations of 12-hydroxyicosa-5,8,10,14-tetraenoic acid (HETE) (Abcam), PGE_2, $PGF_{2\alpha}$ and Tbx B_2 (Cayman Chemical Company) were measured by ELISA as instructed by the manufacturer.

2.7 Statistical analysis

Statistical analysis was carried out using SPSS (Version 21.0, IBM, Armonk, NY: IBM Corp.). Data from dose-response experiments was analysed by 1-way ANOVA with Dunnett's *post hoc* test to compare the effect of each concentration of inhibitor to control. Experiments involving single concentrations of inhibitors were analysed by Student's unpaired t test. Statistical significance was assumed at probabilities less than 0.05. The sample size of ten replicates was sufficient to detect a 10% difference in the primary outcome measures; mRNA expression and synthesis of deuterated fatty acids, with a statistical power of 85% and probability of $P < 0.05$ [12,29].

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Results

3.1 n-6 PUFA biosynthesis

18:2n-6, 20:3n-6, 20:4n-6 and 22:4n-6 were the major [^{13}C]-labelled fatty acids in MOVAS and in Hepa1-6 cells (Figure 1A). The amount of [^{13}C]18:2n-6 was 1.8 fold greater ($P = 0.009$) in Hepa1-6 cells compared to MOVAS cells. Incorporation of [^{13}C] into 20:4n-4 was 2.4-fold ($P=0.04$) and in to 22:4n-6 was 2.7-fold ($P=0.02$) greater in MOVAS cells than Hepa1-6 cells, but the enrichment in 20:3n-6 did not differ between cell types (Figure 1A). Calculation of product to precursor ratios as proxy measures of enzyme activities for individual reactions indicated that conversion of 18:2 to 20:3n-6 (marking combined D6d and elongase-5 activities) was significantly greater in MOVAS cells compared to Hepa1-6 cells (Figure 1B). The ratio of 20:4n-6 to 20:3n-6 (marking D5d activity) was also significantly greater (2.5-fold) in MOVAS cells compared to Hepa1-6 cells (Figure 1B). However, there was no significant difference between cell types in the ratio of 22:4n-6 to 20:4n-6 (marking elongase-2 and/or 5 activities). No incorporation of [^{13}C] into 22:5n-6 above background was detected. There was no significant effect of treating MOVAS cells with Pe on [^{13}C] enrichment of individual PUFA (Figure 1A).

3.2 mRNA expression of Fads and Elovl genes

Fads1 and 2, and Elovl 5 mRNA expression was detected in mouse and rat aorta, in MOVAS cells and in primary HASM cells (Figure 2 A). Because of potential differences in RTPCR primer efficiency, comparisons of mRNA expression of each of the transcripts were only possible within a species. There were significant differences in Fads1 and Fads2 expression between mouse-derived cells and

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tissues (ANOVA both P<0.0001). Fads1 and Fads2 mRNA expression was higher in MOVAS and Hepa1-6 cells than in the corresponding primary tissue (Figure 2A). However, there were no significant differences in Fads1 or Fads2 expression between MOVAS and Hepa1-6 cells or between mouse aorta and liver. Elovl5 expression differed between mouse-derived tissues (ANOVA P = 0.0002). The level of Elovl5 was lower in mouse aorta than in MOVAS cells, but was similar in Hepa1-6 cells and liver. Elovl5 expression in Hepa1-6 cells and liver was lower than in MOVAS cells and higher than in aorta. Elovl2 was not detected in either mouse or rat aorta, or in MOVAS or HASM cells (Figure 2A-C), but was readily detected in Hepa1-6 cells, and in mouse and rat liver (Figure 2A,B).

3.3 DNA methylation of the 5'-regulatory region of Elovl2

In order to determine whether the silencing of Elovl2 in vascular-derived cells was due to the methylation status of its promoter, twenty-nine CpG loci were measured in a region between -117 and -368 bp from the transcription start of the gene (Figure 3). There were significant differences in methylation of three CpG loci between mouse aorta and liver (Figure 3A). Eleven CpG loci had lower methylation in MOVAS cells than in Hepa1-6 cells in the region between -368 and -212bp from the Elovl2 transcription state site (Figure 3B). Conversely, six CpG loci had higher methylation in MOVAS cells than in Hepa1-6 cells in the region between -135 and -117 bp from the Elovl2 transcription start site (Figure 3B). There were no significant differences in the level of methylation between MOVAS and Hepa1-6 cells in the intervening region between -208 and -156 bp. There were marked differences in the overall pattern of DNA methylation of the Elovl2 5'-regulatory region between aorta and liver, and MOVAS and Hepa1-6 cells (Figure 3A,B). In aorta and liver, the level

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of methylation of the CpG loci in the region that was measured was relatively low, and below the detection limit of the assay for the majority of loci. Conversely, in MOVAS and Hepa1-6 cell, the region between -368 and -239 bp was highly methylated compared to aorta and liver. The level of methylation of the region between -135 and -117 bp was markedly greater in MOVAS cells, but not Hepa1-6 cells, than in aorta or liver.

3.4 The effect of D6d or D5d inhibition on Pe-mediated calcium release in MOVAS cells

Both SC26196 and sesamin reduced Pe-mediated calcium release compared to the DMSO vehicle (both ANOVA $P<0.0001$). SC26196 reduced Pe-mediated calcium release in a dose-related manner by 19% (0.5 µmol/l) and by 33% at 1.0 µmol/l in MOVAS cells (Figure 4A). Similarly, treatment with sesamin (10 µmol/ and above**)** reduced Pe-mediated calcium release by 32% (Figure 4B).

3.5 The effect of D6d or D5d inhibition on Pe-mediated eicosanoid release in MOVAS cells

Treatment of MOVAS cells with SC26196 induced a statistically significant, concentration-related decrease in Pe-mediated secretion of PGE_2 and $PGF_{2\alpha}$ (ANOVA $P = 0.0152$ and $P = 0.008$, respectively) such that compared to Pe alone, treatment with 1µmol/l SC26196 reduced PGE_2 concentration by 53% and $PGF_{2\alpha}$ concentration by 39.3% (Figure 5 A,B). In contrast, there was no significant effect of treatment with SC26196 on Pe-mediated 12-HETE or Tbx B2 secretion (Figure 5 C,D).

Treatment of MOVAS cells with sesamin induced a statistically non-significant, concentration-related trend towards lower PGE_2 secretion ($P= 0.078$) and

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a statistically significant (P = 0.007) decrease in $PGF_{2\alpha}$ secretion (Figure 5 E,F). Compared to Pe alone, treatment with 20 μmol/l sesamin reduced PGE_2 concentration by 37% and $PGF_{2\alpha}$ concentration by 67% (Figure 5 A,B). There was no statistically significant effect of treatment with sesamin on Pe-mediated 12-HETE or Tbx B2 secretion (Figure 5 G,H).

4. Discussion

4.1 Characterisation of n-6 PUFA biosynthesis in VSM cells

The present findings show that VSM cells are able to synthesise a range of longer chain, more unsaturated fatty acids from 18:2n-6 and that PUFA biosynthesis is involved in Pe-mediated release of calcium from intracellular stores and production of specific eicosanoid species.

We have shown previously that inhibition of D6d and D5d activity in *ex-vivo* preparations of rat aortae and in human femoral arteries reduced α_1AR-mediated vasoconstriction and the secretion of specific pro-vasoconstrictor eicosanoids [12]. Furthermore, PUFA biosynthesis activity associated with vasoconstriction was localised to VSM cells rather than the vascular endothelium [12]. In order to investigate further the function of PUFA biosynthesis in vasoconstriction, we characterised the activity of this pathway in VSM cells *in vitro*. The findings of the present study support our previous observations by demonstrating that MOVAS cells converted 18:2n-6 to longer chain PUFA, including the longest chain metabolite of the n-6 PUFA series 22:5n-6. This is in agreement with the single previous report of n-6 PUFA metabolism in VSM cells from human aortae [8]. Previous studies have shown that 18:2n-6 can be converted to 20:4n-6 in human umbilical vein endothelial cells *in vitro* [6,7]. However, the extent of conversion appears to be limited to 20:4n-

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6 with no detectable synthesis of longer chain metabolites [6]. Overall, these findings are consistent with differential function of PUFA biosynthesis between vascular endothelial and smooth muscle cells.

4.2 mRNA expression of genes involved in PUFA biosynthesis and DNA methylation of Elovl2

The expression of Fads1 and Fads2 was similar in MOVAS and Hepa1-6 cells and in mouse aortae and liver, although the levels of these transcripts were lower in the tissues compared to the cell lines. However, synthesis of 20:4n-6 and 22:4n-6 was greater in MOVAS cells than in Hepa1-6 cells. Thus the level of PUFA biosynthesis involves additional regulatory factors controlling transcription and/ or translation. Elovl5 expression was markedly higher in MOVAS compared to aortae, but similar to Hepa1-6 cells and in liver. This implies that PUFA biosynthesis activity in the MOVAS cells and in Hepa1-6 cells may not be completely representative of aortae and liver, respectively. However, despite this difference, PUFA biosynthesis may be at least as active in VSM as in liver. PUFA biosynthesis is highly active in liver as it provides substrates for cell membrane and lipoprotein synthesis such that Fads2 and Fads1 mRNA expression is markedly higher in liver compared to other tissues, with the exception of testis [3,30]. If so, one possible implication is that the PUFA biosynthesis contributes to other processes in VSM in addition to maintaining cell membrane composition.

Elovl2 mRNA was not detected in MOVAS cells, rat aortae or primary HASM cells, but was detected readily in Hepa1-6 cells, and in mouse and rat liver. One interpretation of this finding is that conversion of 20:4n-6 to 22:4n-6 and 22:4n-6 to 24:4n-6 involves another elongase, although we were not able to identify which

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enzyme catalyses these reactions. However, the possiblity that Elovl2 was expressed at a level below the limit of the RTPCR assay cannot be discounted completely. The reason for the apparent differential use of elongase 2 between VSM and liver is not known. It is possible that the use of an alternative elongase is important for the regulation of PUFA biosynthesis related to a specific function in VSM.

DNA methylation of the 5'-regulatory regions (5'-RR) of genes is associated generally with suppression of transcription [31]. We investigated whether the 5'-RR of Elovl2 was hypermethylated in vascular cells compared to liver. The findings show that the level of methylation of 5'-RR of Elovl2 was close to the lower limit of detection of the sequencing technique (5%) [12]. Thus Elovl2 was essentially unmethylated in both mouse aortae and in liver. The pattern of DNA methylation of Elovl2 5'-RR was similar in MOVAS and Hepa1-6 cells in the region between -638 and -156 bp, although methylation of CpG loci in the sequence furthest from the transcription start site was higher in Hepa1-6 compared to MOVAS cells. In contrast, methylation of CpG loci in the most proximal region was higher in MOVAS compared to Hepa1-6 cells. However, the pattern of DNA methylation of MOVAS and Hepa1-6 cells differed markedly from aortae and liver, respectively, which may represent an effect of adaptation to *in vitro* culture. Together, these findings suggest that suppression of Elovl2 transcription in VSM cells and in aortae was not associated with hypermethylation of the gene, although it is possible that sequences either closer to the TSS or more distal to region that was analysed could have been be differentially methylated.

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4.3 The role of PUFA biosynthesis in intracellular calcium release and in eicosanoid secretion in VSM cells

SC26196 and sesamin are structurally distinct, specific inhibitors of D6d and D5d, respectively [8,21,22]. As expected, treatment of MOVAS cells with SC26196 increased the proportion of 18:2n-6 and decreased the proportions of all down-stream metabolites in total cell lipids, consistent with specific inhibition of D6d, which is consistent with Harmon *et al*. [8]. Furthermore, treatment with sesamin increased the proportions of 18:2n-6, 18:3n-6 and 20:3n-6 in total cells lipids which is consistent with specific inhibition of D5d [22]. Together these findings show that SC26196 and sesamin are selective inhibitors of PUFA biosynthesis in VSM cells.

Both SC26196 and sesamin induced a concentration-related inhibition of Pe-mediated PGE_2 and $PGF_{2\alpha}$ secretion, although this did not reach statistical significance for $PGF_{2\alpha}$ in cells treated with sesamin. However, there was no significant effect of these inhibitors on the secretion of 12-HETE or $TbxA_2$ (measured as it stable degradation production $TbxB_2$). These results are consistent with our previously observations that inhibition of D6d by SC26196 in rat aortae reduced the secretion of the pro-vasoconstrictor eicosanoids PGE_2, $PGF_{2\alpha}$ and $TbxB_2$, although there was no significant effect on 12-HETE secretion [12]. However, the present results extend these findings by showing that inhibition of D5d has similar effects on PGE_2 and $PGF_{2\alpha}$ secretion as D6d inhibition. PGE_2 can be converted to $PGF_{2\alpha}$ by prostaglandin E 9-ketoreductase activity, as well as direct synthesis of both PGE_2, and $PGF_{2\alpha}$ from PGH_2. Thus possible mechanisms by which inhibition of D6d or D5d may reduce PGE_2, and $PGF_{2\alpha}$ secretion is by reduction in the availability of 20:4n-6 for synthesis of PGH_2 by cyclooxygenase activity. If so, it would be expected that TbxB2 secretion would also be reduced. Our previous experiments

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that used mass spectrometry to analyse supernatants from *ex vivo* preparations of rat aortae stimulated with Pe showed that inhibition of D6d reduced TbxB2 concentration [12] and so it is possible that the ELISA assay used here was not sufficiently sensitive to detect TbxB2 in culture supernatants from Pe-stimulated MOVAS cells. 12-HETE is produced by 5-lipoxygenase activity. Thus one possible explanation for the differential effect of inhibition of PUFA biosynthesis by SC26196 and sesamin is that 20:4n-6 synthesised *de novo* is used preferentially by cyclooxygenase, compared, for example, to 20:4n-6 derived pre-formed from the culture medium.

SC26196 reduced Pe-mediated calcium release in a concentration-related manner. Furthermore, sesamin also inhibited Pe-mediated calcium release. These findings are consistent with our previous observations that PUFA biosynthesis *de novo* in VSM cells is involved centrally in α_1AR-mediated vasoconstriction [12]. However, the present findings extend the previous observations by demonstrating the involvement of PUFA biosynthesis in intracellular calcium release. Although both SC26196 and sesamin have been shown to inhibit D6d and D5d specifically [8,21,22] it is possible that these inhibitors may have reduced calcium release via an effect on another pathway. However, the structural differences between SC26196 and sesamin suggest that it is unlikely, although further experiments are required to provide conclusive findings.

Contraction of VSM involves release of calcium from internal stores as a result of the activation of G protein receptors leading to calcium sensitisation [32,33]. This process also involves inhibition of MLCP [14] by a mechanism involving non-esterified 20:4n-6. The present findings are consistent with these observations, but extend these findings by showing that 20:4n-6 synthesised *de novo* is involved in Pe-

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mediated calcium sensitisation. Thus we suggest cautiously that one interpretation is that 20:4n-6 synthesised *de novo* may be the principle source of 20:4n-6 that inhibits MLCP and acts a substrate for the synthesis of pro-vasoconstriction eicosanoids that induce intracellular calcium release (Figure 6). However, this model requires further investigation.

Impaired vasodilatation and increased vasoconstriction are causal processes in a number of pathological conditions of major health importance and involve impaired regulation of calcium sensitisation [34]. Identification of a novel novel component of the pathway that regulates release in VSM cells raises the possibility of new therapeutic interventions in vascular disease.

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Figure 1. Synthesis of n-6 PUFA in MOVAS and Hepa1-6 cells

Values are mean SEM for n=10 replicate cultures. (A) Incorporation of [^{13}C] into n-6 PUFA in MOVAS cells in the absence or presence of Pe, or into Hepa1-6 cells. Statistical analysis was by 1-way ANOVA with Dunnett's post hoc pairwise test. (B) Product to precursor ratios of [^{13}C]-labelled n-6 PUFA in MOVAS and Hepa1-6 cells representing proxy measures of; D6d plus elongase 5 activities (20:3n-6/18:2n-6), (D5d activity 20:4n-6/20:3n-6) or D5d plus elongase 2 or 5 activities (22:4n-6/20:3n-6). The effect of treatment with (C) SC26196 (200nM) or (D) sesamin (20μM) on the

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n-6 PUFA content of MOVAS cells. Panels B-D, means that were statistically significantly different from control cultures by Student's t test are indicated by *P<0.05, **P<0.01, ***P<0.001.

Figure 2. mRNA expression of genes involved in PUFA biosynthesis. Values are mean ± SEM (n=10 replicates). (A) Mouse cells and tissues, (B) rat tissues and (C) human primary vascular smooth muscle cells. Panel (A), means that were statistically significantly different (P<0.05) from MOVAS cells for each genes by 1-way ANOVA with Dunnett's *post hoc* pairwise comparisons, except for Elovl2 where Hepa1-6 cells and liver were compared by Student's unpaired t test, are indicated by different letters. Panel B, means that were significantly different between cells and tissues by Student's unpaired t test are indicated by *P<0.05, **P<0.01, ***P<0.001. Panel (C), no statistical analysis was carried out.

Figure 3. Methylation of individual CpG loci in the 5'-regulatory regions of Elovl2. Values are mean ± SEM (n=10 replicates). (A) mouse tissues and (B) mouse cells. CpG loci are indicated relative to the transcription start site (TSS). Dotted lines indicate the limited of detection. Means that were significantly different between tissues or between cell types by Student's unpaired t test are indicated by *P<0.05, **P<0.01, ***P<0.001.

Figure 4. The effect of inhibition of delta-6 or delta-5 desaturase on phenylephrine-mediated calcium release in MOVAS cells. Values are mean ± SEM (n=10 replicates). (A) Treatment with SC26196, (B) treatment with sesamin. Means that were significantly different from vehicle alone by 1-way ANOVA with Dunnett's post hoc test are indicated by *P<0.05, **P<0.01, ***P<0.001.

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Figure 5. The effect of treatment with or sesamin on the phenylephrine-mediated secretion of PGE_2, $PGF_{2\alpha}$, $TbxB_2$ and12-HETE. Values are mean ± SEM (n=10 replicates). Means that were significantly different from vehicle alone by 1-way ANOVA with Dunnett's post hoc test are indicated by *P<0.05, **P<0.01, ***P<0.001.

Figure 6. A model for the role of PUFA biosynthesis *de novo* in calcium sensitisation in vascular smooth muscle cells. A detailed explanation is presented in the text. $aPKC_z$, atypical protein kinase C-zeta; $ciPLA_2$, calcium-indpendent phospholipase A_2; D5d, delta-5 desaturase; D6d, delta-6 desaturase; MLCP, myosin light chian phosphatase. GO = activation,  = inhibition.

HIGHLIGHTS

Vasoconstriction involves polyunsaturated fatty acid synthesis in vascular smooth muscle cells.

We investigated the underlying mechanism in vascular smooth muscle (VSM) cells in vitro.

Δ6 or Δ5 desaturase inhibition reduced phenylephrine-induced calcium release.

Δ6 or Δ5 desaturase inhibition also reduced PGE_2, and $PGF_{2\alpha}$ secretion.

We propose a model for the role of PUFA biosynthesis in calcium sensitisation in VSM.

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Table 1. Real time RTPCR primers

Gene	Forward primer	Reverse primer
Human PPIA (Cyclophillin A)	Qiagen Hs_PPIA_1_SG QuantiTect primer Assay (QT00052311)	
Human D5d	5'-CGCAAGGTTTACAACATCAC-3'	5'-GCCAGTTCACCATCAGC-3'
Human D6d	5'-CGCAAGGTTTACAACATCAC-3'	5'-GCCAGTTCACCATCAGC-3'
Human Elongase 2	Qiagen Hs_ELOVL2_1_SG QuantiTect primer Assay (QT00059017)	
Human Elongase 5	Qiagen Hs_ELOVL5_1_SG QuantiTect primer Assay (QT00096334)	
Mouse PPIA (Cyclophillin A)	Qiagen Mm_Ppia_1_SG QuantiTect primer assay (QT00247709)	
Mouse D5d	Qiagen Mm_Fads1_1_SG QuantiTect primer assay (QT00114184)	
Mouse D6d	Qiagen Mm_Fads2_1_SG QuantiTect primer assay (QT00130018)	
Mouse Elongase 2	Qiagen Mm_Elovl2_1_SG QuantiTect primer assay (QT00091644)	
Mouse Elongase 5	Qiagen Mm_Elovl5_1_SG QuantiTect primer assay (QT00117705)	
Rat PPIA(Cyclophillin A)	Qiagen Rn_Ppia_1_SG QuantiTect Primer Assay (QT00177394)	
Rat D5d	Qiagen Rn_Fads1_1_SG QuantiTect primer assay (QT00188664)	
Rat D6d	Qiagen Rn_Fads2_1_SG QuantiTect primer assay (QT00186739)	
Rat Elongase 2	Qiagen Rn_Elovl2_2_SG QuantiTect primer assay (QT01683899)	
Rat Elongase 5	Qiagen Rn_Elovl5_va.1_SG QuantiTect primer assay (QT02466653)	

The sequences used in QuantiTect primer assays (Qiagen, West Sussex, UK) are commercial property and were not made available by the company. All other primers were from Biomers (Söflinger, Germany).

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Table 2 PCR and pyrosequencing primers for analysis of the 5'-regulatory region of Elovl 2

CpGs	Forward primer (5' to 3')	Reverse primer (3' to 5')	Amplicon bp)
-386, -346,-343, -341, -337, -313	AGTGTTGGGATTATATAGGTGTATTAAT T	AAACCACCAACAACTCCT	141
-266, -262, -260, -239	AGTGTTGGGATTATATAGGTGTATTA	AAACCCCCCAAAAACCTA	297
-212, -208, -198, -195, -193, -187, -184, -184, -181, -173, -171, -169, -164, -158, -156	AGTGTTGGGATTATATAGGTGTATTA	AAACCCCCCAAAAACCTA	297
-135, -129, -127, 123, -120, -117	GTAGGTTTTTGGGGGGTTT	CCCCCACTCACTCACCAT	272
	Sequencing primers		
-386, -346,-343, -341, -337, -313		GATTAGTTTTTGGTTATTTTTTTAA	
-266, -262, -260, -239		GAGTTTGATGTTGTGATAGA	
-212, -208, -198, -195, -193, -187, -184, -184, -181, -173, -171, -169, -164, -158, -156		GTTAGTTGTGTTTGTAAATTTTA	
-135, -129, -127, -123, -120, -117		GGTTTTTGGGGGGTTTA	

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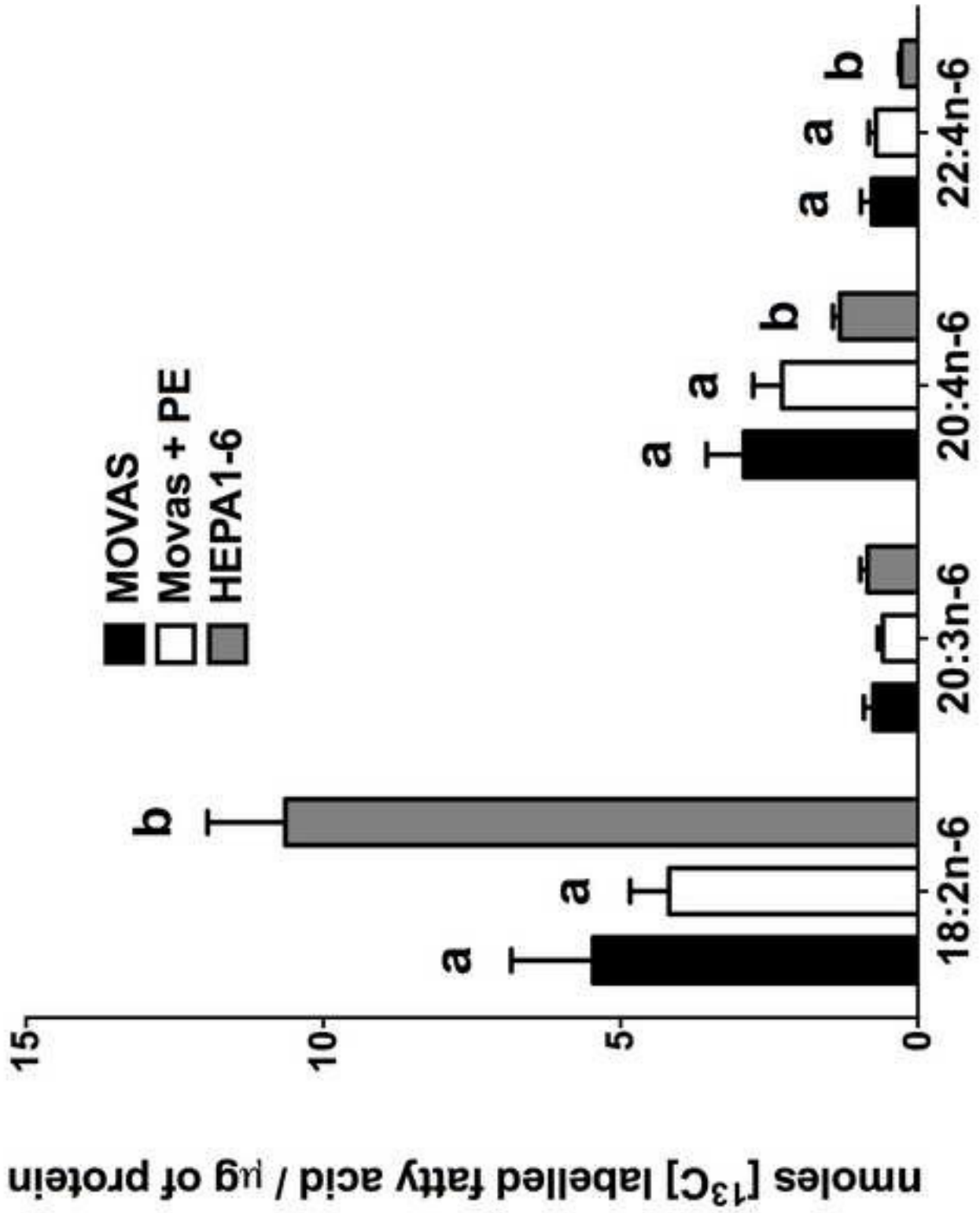

Figure 1

Figure 2

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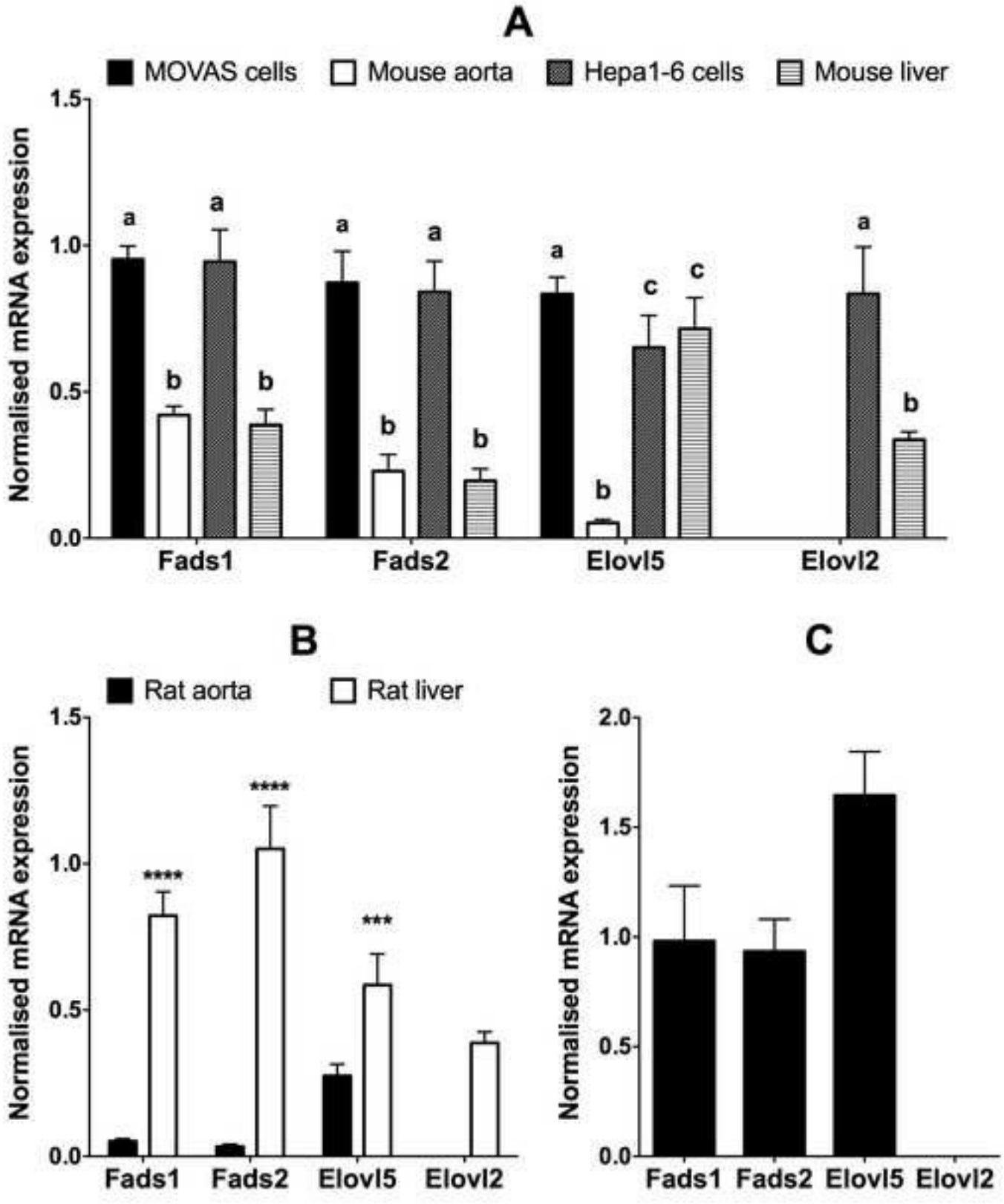

Figure 3

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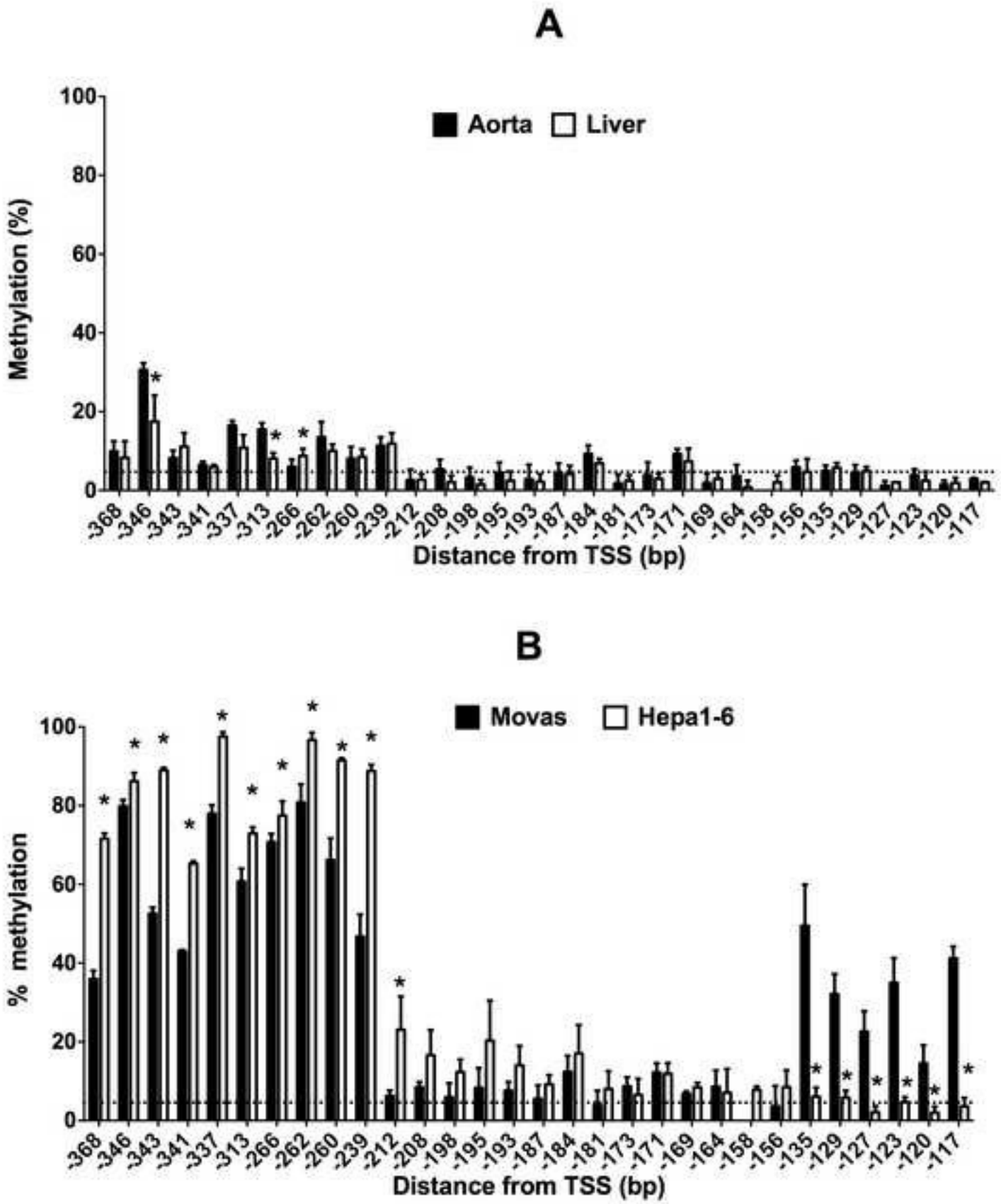

Figure 4

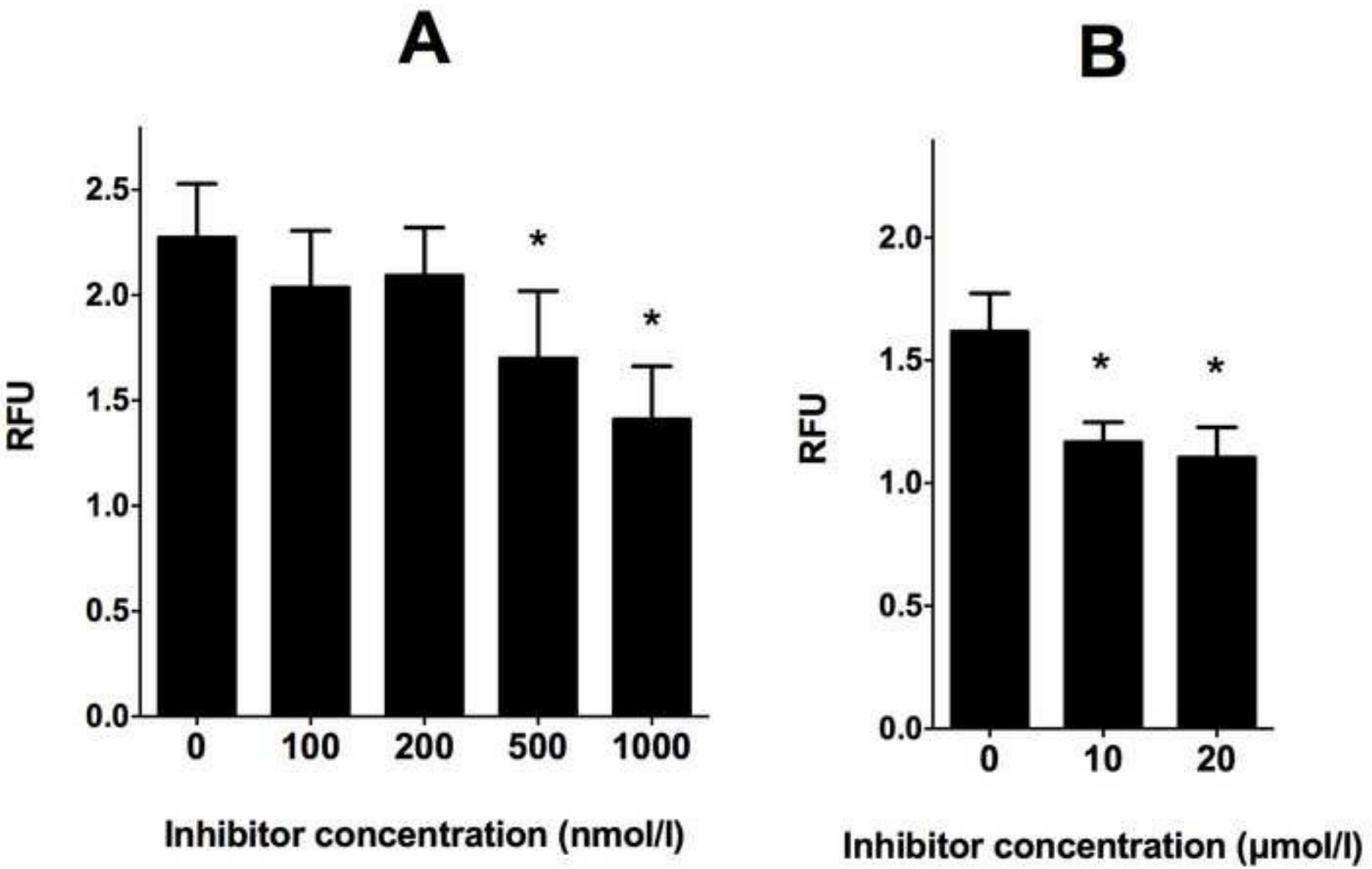

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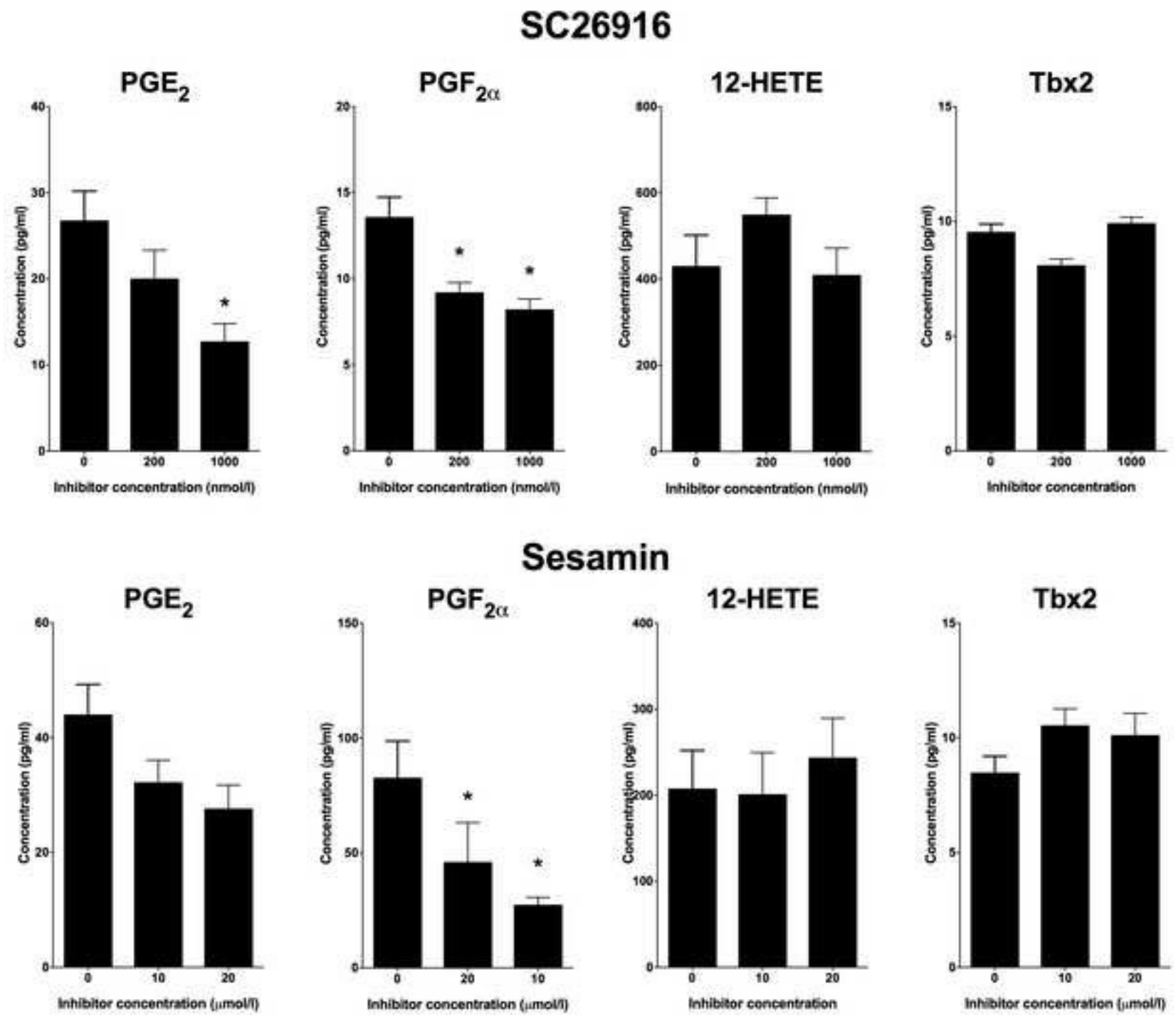
Figure 5



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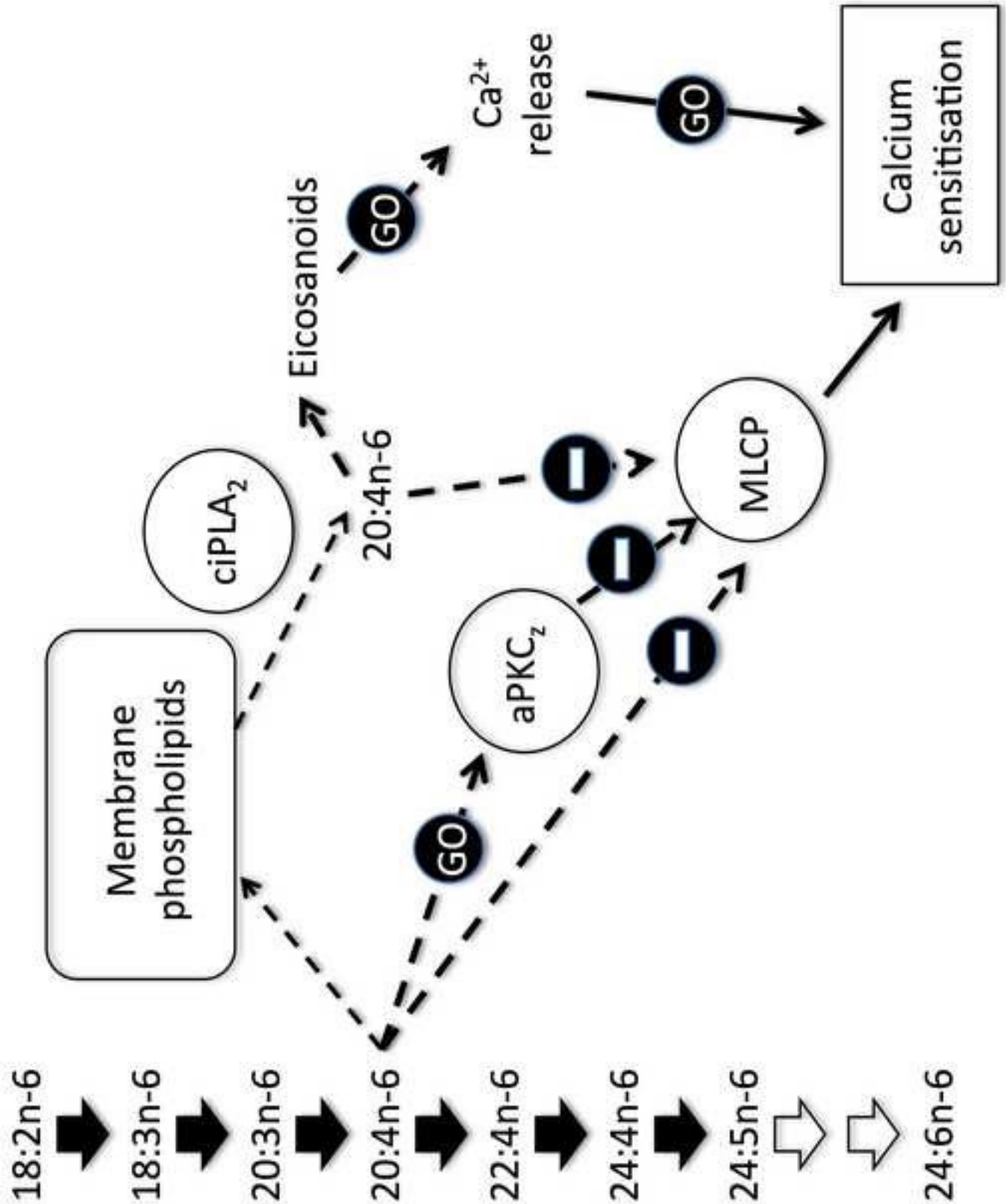

Figure 6