



A PPAR-independent pathway to PUFA-induced COX-2 expression

K. Derecka, E.L. Sheldrick, D.C. Wathes, D.R.E. Abayasekara, A.P.F. Flint

► To cite this version:

K. Derecka, E.L. Sheldrick, D.C. Wathes, D.R.E. Abayasekara, A.P.F. Flint. A PPAR-independent pathway to PUFA-induced COX-2 expression. *Molecular and Cellular Endocrinology*, 2008, 287 (1-2), pp.65. 10.1016/j.mce.2008.02.015 . hal-00532004

HAL Id: hal-00532004

<https://hal.science/hal-00532004>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: A PPAR-independent pathway to PUFA-induced COX-2 expression

Authors: K. Derecka, E.L. Sheldrick, D.C. Wathes, D.R.E. Abayasekara, A.P.F. Flint



PII: S0303-7207(08)00087-7
DOI: doi:10.1016/j.mce.2008.02.015
Reference: MCE 6824

To appear in: *Molecular and Cellular Endocrinology*

Received date: 5-10-2007
Revised date: 15-2-2008
Accepted date: 16-2-2008

Please cite this article as: Derecka, K., Sheldrick, E.L., Wathes, D.C., Abayasekara, D.R.E., Flint, A.P.F., A PPAR-independent pathway to PUFA-induced COX-2 expression, *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2008.02.015

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A PPAR-independent pathway to PUFA-induced COX-2 expression

K. DERECKA, E. L. SHELDRIK, D. C. WATHES^a, D. R. E. ABAYASEKARA^a, AND
A.P.F. FLINT¹

*Division of Animal Physiology, School of Biosciences, University of Nottingham, Sutton Bonington
Campus, Loughborough, Leics LE12 5RD, UK and ^aReproduction, Genes and Development Group,
Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, UK*

Running title: PUFAs, PPARs and COX-2

¹Corresponding author: Division of Animal Physiology, School of Biosciences, University of
Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK. Tel.: +44 115
9516301; fax: +44 115 9516302. *Email address:* Anthony.Flint@nottingham.ac.uk

Keywords: Arachidonic acid; Protein kinase C; Peroxisome-proliferator-activated receptor; Cyclooxygenase 2; (Bovine endometrium).

Summary

Polyunsaturated fatty acids (PUFAs) induce COX-2 in bovine endometrial stromal cells through activation of peroxisome-proliferator-activated receptor alpha (PPAR α). We have investigated alternative (PPAR-independent) pathways to COX-2 induction using a reporter construct driven by a *COX-2* gene promoter sequence lacking a PPAR response element. This construct was induced by PUFAs, but not by PPAR agonists. PPAR-independent reporter gene expression occurred 6 h after PPAR-dependent induction of the endogenous *COX-2* gene. In contrast to PPAR-dependent COX-2 induction, which is not affected by NF- κ B inhibitors, the PPAR-independent pathway was blocked by the NF- κ B inhibitor MG132 or following deletion of NF- κ B sites in the *COX-2* promoter. The PPAR-independent effect of PUFA was mimicked by the PKC activators 4 β -PMA and prostaglandin F_{2 α} , but was not blocked by the PKC inhibitor RO318425. The results demonstrate a pathway to the induction of COX-2 by PUFAs requiring NF- κ B but not PPAR or PKC.

INTRODUCTION

Various hormones interact with G-protein-linked cell surface receptors to activate phospholipases and raise intracellular levels of polyunsaturated fatty acids (PUFAs). A good example is the effect of oxytocin exerted via oxytocin receptors on the epithelium of the uterine endometrium, which leads to activation of phospholipases C and A, releasing arachidonic acid and other PUFAs esterified in phospholipids (Flint et al., 1986). PUFAs produced in this way may then act as second messengers, both through activation of protein kinases and via other metabolic products (Khan et al., 1995; Maloberti et al., 2005).

PUFAs may also be secreted by the cells in which they are generated, to act on neighbouring cells as paracrine messengers (Cooke et al., 1991; Ronco et al., 2002). This has been suggested to occur in the endometrium in relation to both activation of cyclic AMP phosphodiesterase (Cheng et al., 2007) and induction of cyclooxygenase 2 (COX-2; also known as prostaglandin endoperoxide synthase 2; Sheldrick et al., 2007). Evidence for an effect on COX-2 includes the observations that PUFAs raise COX-2 levels in rat uterine stromal cells (Prigent-Tessier et al., 1996) and in bovine endometrial epithelial cells (Parent et al., 2003) through increased *COX-2* gene expression and increased levels of COX-2 transcripts (Asselin et al., 1997; Meade et al., 1999). Furthermore the oxytocin receptor is expressed in the bovine endometrial epithelium at a time when it is not expressed in the stroma (Robinson et al., 1999). Arachidonic acid has therefore been proposed to act as a paracrine signal released during the oestrous cycle by epithelial cells to induce COX-2 in the stroma (Sheldrick et al., 2007). The experiments described here were carried out to investigate the pathways by which PUFAs induce *COX-2* gene expression in endometrial stromal cells.

Previous experiments on the mechanism by which arachidonic acid raises COX-2 levels suggest that peroxisome-proliferator-activated receptors (PPARs) mediate PUFA-induced COX-2 expression in the bovine endometrium (MacLaren et al., 2006; Sheldrick et al., 2007). The isoform PPAR α appears to be involved in bovine endometrial stromal cells. PPAR α is activated directly by a range of PUFAs, including arachidonic acid. However, PPAR α function is dependent on phosphorylation by protein kinase C (PKC; Blanquart et al., 2004), which is also activated by PUFAs (Shinomura et al., 1991; Khan et al., 1995). Furthermore, in some cells PKC induces COX-2 through a second pathway, involving NF- κ B (Paik et al., 2000; Wu

and Wiltbank, 2001; Yan et al., 2002). As a result arachidonic acid may potentially induce COX-2 directly via PPAR α and indirectly through PKC/NF- κ B.

To distinguish between PPAR-dependent and -independent pathways we have used a reporter construct under the control of a naturally-occurring sequence derived from the bovine *COX-2* promoter. This construct, which includes most of the important transcription regulators present in the *COX-2* promoter but lacks an identifiable peroxisome-proliferator response element (PPRE), allows the study of pathways to *COX-2* induction in which PPARs are not involved. Furthermore we have investigated the role of NF- κ B in the function of the promoter by sequential removal of the NF- κ B sites from this construct.

MATERIALS AND METHODS

Materials

All PUFAs; synthetic PPAR agonists; the NSAIDs, indomethacin and NS398; prostaglandin A₁ (PGA₁); the phorbol ester 4 β -PMA and its inactive analogue 4 α -phorbol 12,13-didecanoate (4 α -PDD); the protein kinase C inhibitor, RO 318425, and the NF- κ B inhibitor, MG132 were obtained from either Sigma (Poole, UK) or Calbiochem (Nottingham, UK). Conjugated linoleic acid (Sigma catalogue number O 5507) was a mixture of cis- and trans-9,11- and -10,12-octadecadienoic acids. The PUFAs, indomethacin and prostaglandins were dissolved in ethanol; all other compounds were added to culture medium in DMSO. Vehicle controls were used as appropriate. PUFAs were stored in ethanol under N₂ at -20° C in darkness.

Cell culture

Bovine endometrial stromal cells isolated on day 16 of the oestrous cycle (Flint et al., 2002) were maintained as described previously (Sheldrick et al., 2007). To test effects of PUFAs on *COX-2* promoter function, the fatty acids were added to culture medium for up to 48 h after transfection.

COX-2 reporter plasmids

A bovine *COX-2* promoter fragment was cloned by PCR on the basis of the genomic sequence (GenBank accession no. [AF031699](#); Liu et al., 1999). The PCR product was sequenced to confirm its identity, and inserted into the vector pCAT promoter 3 (Promega, Southampton,

UK). The resulting construct was designated pCATCOXprom. This promoter sequence did not contain a PPRE (which is further upstream of the transcription start site; Meade et al., 1999). NF- κ B mutants (Lee et al., 2001) were prepared from pCATCOXprom by site directed mutagenesis using the QuikChange multi site-directed mutagenesis kit (Stratagene, Cambridge, UK) and checked by sequencing. The NF- κ B consensus sequence GGGAATTCCC at -472 (numbering from the transcription start site) and the related sequence GGAACGTCCC at -454 were both mutated to GGCCTTCCC in separate plasmids (designated Del I and Del II respectively). A third plasmid (Del I/II) contained both mutated sequences. All deletions were confirmed by sequencing. pCAT basic and pCAT control vectors (Promega) were used as controls.

Transient transfections

Stromal cells at 50-60% confluence were washed and trypsinized before resuspension in Dulbecco Modified Eagles Medium (DMEM; Sigma, Poole, UK). They were then electroporated at 300 volts, 1650 μ F with 5 μ g plasmid DNA in a volume of 0.8 ml DMEM, at a concentration of $1-3 \times 10^6$ cells per ml (conditions which had been optimized in preliminary experiments). Electroporated cells were suspended in DMEM containing 1% antibiotic-antimycotic and 10 % fetal bovine serum previously stripped with dextran-coated charcoal (5% w/v; DMEMSS) and transferred to 24-well plates ($1.0-1.5 \times 10^5$ cells per well). Test compounds were then added to the medium as required, and culture continued for 36 or 48 h. For time course experiments compounds were added at various times before the end of culture; thus in the case of cells treated for 3 h, compounds were added 3 h before culture was terminated at 48 h after medium change. At the end of culture cells were washed once in 1 ml ice-cold phosphate-buffered saline containing 0.2 mM sodium orthovanadate and lysed for 20 min on ice with reporter lysis buffer (100-200 μ l; Promega) for assay of chloramphenicol acetyl transferase and protein.

Chloramphenicol acetyl transferase assay

Chloramphenicol acetyl transferase (CAT) was measured in cell lysates by a 2 phase method. Pre-cleared cell lysates (30 μ l) were incubated at 37° C with 10 μ l 5 mM chloramphenicol in 250 mM Tris containing 5 mM EDTA, pH 7.8 in 96-well plates. After 5 min, 10 μ l 75 μ M HCl containing 1.5 nmol [3 H]acetylcoenzyme A (Amersham, Little Chalfont, UK) was added to each tube. The plates were incubated at 37° C for 3 h. The reaction mixture (48 μ l) was

transferred to scintillation vials containing 0.5 ml 7 M urea and 5 ml 0.8% w/v toluene-PPO was added to each vial. The vials were capped and shaken for 15 sec. [³H]Acetylchloramphenicol extracted into the toluene out of the aqueous phase was measured by scintillation counting after 20 min.

Immunoblotting

Cells were lysed and extracted for immunoblotting as described previously (Sheldrick et al., 2007). For immunoblotting, boiled lysates (10 µg protein) were subjected to electrophoresis on 10% acrylamide gels (5% stacking gels) before electroblotting onto Protran BA83 (Schleicher & Schuell, Anderman and Company Kingston-upon-Thames, Surrey, UK) membranes in 25 mM Tris pH 8.3 containing 148 mM glycine and 20% (v/v) methanol. For detection of COX-2 bands (at molecular mass = 72 kDa), membranes were probed with COX-2 antibody (C-20; SC 1745, Santa Cruz Biotechnology, obtained through Autogen Bioclear, Calne, UK; 1:250 dilution in a solution containing 1% (w/v) Marvel milk powder and 0.5% (w/v) Tween 20 in phosphate buffered saline). Second antibody was donkey anti-goat IgG-horseradish peroxidase (SC 2020; Santa Cruz Biotechnology; 1:11 000 dilution in 3% (w/v) Marvel, 0.5% (w/v) Tween 20 in phosphate buffered saline), and visualization was by enhanced chemiluminescence (Amersham) using Kodak BioMax Light film. Colour markers (molecular masses 29 000 – 205 000; Sigma) were used to identify molecular masses of proteins. Band intensities were quantified using Kodak 1D digital image analysis software.

Protein assay

Protein concentrations in lysates prepared for electrophoresis were measured before the addition of β-mercaptoethanol and bromophenol blue using BCA reagents (Perbio Science, Cramlington, UK; Bradford, 1976).

Experimental design and analysis

Experimental treatments were carried out in 24-well plates, with each replicate treatment of cells in at least 3 wells. Within each experiment all cells were transfected in a single batch before transfer to 24-well plates, and as a result there were no differences between wells in transfection efficiency. Data were therefore expressed in terms of cell protein, and not corrected for transfection efficiency. To account for differences in band intensity between immunoblots, all blots included 2 control samples, and all experimental treatments were related

to the control bands. All bands were included for analysis. Statistical analysis of treatment effects was by analysis of variance using Genstat version 8 (Lawes Agricultural Trust; obtained through VSN International Ltd, Hemel Hempstead, UK). Where significant effects were detected ($P < 0.05$) individual differences were tested using a post-hoc test (Bonferroni's multiple comparison test). Values are given as means \pm s.e.m of results of separate experiments. In the figures significant treatment effects (from controls or zero-time treatments) are indicated by different letters above bars.

RESULTS

Structure of the COX-2 promoter

The nucleotide sequence of the bovine *COX-2* gene promoter is shown in Fig. 1. PPARs form heterodimers with the 9-cis retinoic acid receptor (RXR) to activate target genes by binding to specific response elements (PPREs). PPREs contain two direct repeats of PuGGTCA half-sites separated by one base (usually A) with an additional 4 bases (usually AACT) comprising the 5' part of binding site, giving the consensus sequence AACTAGGNCAAAGGTCA (the hexameric half-sites being italicised; Ijpenberg et al., 1997). The 5' half of this response element is occupied by a PPAR, the 3' half binding the RXR. The bovine *COX-2* promoter region contains a group of potential half-binding sites (Fig. 1), but the bovine equivalent of the human *COX-2* PPRE, which is 3.9 kb upstream of the transcription start site (Meade et al., 1999), was not present. A sequence (AAGAGGGGGAAAAGTTT) resembling the PPRE of human lipoprotein lipase (AAGAGGGGGAAAGGGCA; Ijpenberg et al., 1997) is located -109 bp upstream of the translation start site in the bovine sequence. This has a perfect 5' PPAR binding motif, but differs in the last 5 nucleotides from the consensus RXR binding motif. However, as shown below, none of these motifs conferred responsiveness to PPAR α in stromal cells, possibly because there was insufficient homology with actual PPREs, because there are species differences between bovine and other consensus sequences, or because of the proximity of the site at -109 to the transcription start site.

pCATCOXprom was not responsive to PPAR activation

As anticipated due to the absence of a PPRE, and in contrast to the effects of PPAR agonists on endogenous *COX-2* gene expression (Sheldrick et al., 2007), the *COX-2* promoter was not responsive to the PPAR α agonists ciprofibrate, methylclofenapate, indomethacin, NS398 or prostaglandin A₁ or to the PPAR γ agonist pioglitazone (Fig. 2). Nonetheless arachidonic acid

did induce CAT expression, indicating the presence of a PPAR-independent pathway for COX-2. The response to PUFA varied between experiments in the range 2.0- to 16.2-fold (mean, 6.1-fold; N = 10). In previous experiments measuring endogenous COX-2 protein, ciprofibrate was as effective a PPAR agonist as WY14643, suggesting that the failure to induce CAT expression was unlikely to reflect poor bioavailability.

Time course of effects of PUFAs on CAT expression

Further evidence that the effect of arachidonic acid (Fig. 2) was not due to activation of a PPAR was obtained from time course studies. At concentrations above 10 μ M, arachidonic acid increased CAT expression between 12 and 24 h after addition (Fig. 3a). Similar responses were observed with n-6 PUFAs other than arachidonic acid (Fig. 3b), although these were delayed until 24 – 36 h. In contrast, induction of the endogenous *COX-2* gene (as indicated by immunoblotting) peaked at 6 h after exposure to agonist (Fig. 3a; data of Sheldrick et al., 2007). The lag before expression of CAT relative to induction of the endogenous COX-2 protein did not reflect the time taken by the cells to recover from electroporation, as the PPAR agonist indomethacin raised COX-2 levels within 6 h in cells that had been electroporated (Fig. 3c).

Involvement of PKC and NF- κ B in induction of CAT expression

To further differentiate between PPAR-dependent and -independent effects, two experimental strategies were used to define the roles of PKC and NF- κ B in pathways to COX-2 induction. Firstly, cells transfected with pCATCOXprom were treated with (i) 4 β -PMA (which specifically activates PKC; Nishizuka, 1984), (ii) prostaglandin F_{2 α} (which activates PKC through the FP receptor and phospholipase C; Abayasekara et al., 1993), (iii) the PKC inhibitor RO318425, or (iv) the NF- κ B inhibitor MG132 (Wu et al., 2004). Secondly, cells were transfected with constructs derived from pCATCOXprom but lacking NF- κ B sites.

Expression of pCATCOXprom was induced by 4 β -PMA and prostaglandin F_{2 α} (Fig. 4a). When these compounds were added with arachidonic acid the effects of arachidonic acid and 4 β -PMA or arachidonic acid and prostaglandin F_{2 α} were additive but not synergistic (i.e. there was no statistically significant interaction). The inactive phorbol ester 4 α -phorbol 12,13-didecanoate (2 μ M) was ineffective. Consistent with a role for NF- κ B, the effects of arachidonic acid and 4 β -PMA were both blocked by the NF- κ B inhibitor, MG132 (Fig. 4b). MG132 also blocked induction of CAT expression by PGF_{2 α} (data not shown) and reduced

basal CAT expression when added alone. In contrast to the effect of MG132, the specific PKC inhibitor RO318425 blocked the response to 4 β -PMA (Fig. 4c) but not the response to arachidonic acid (Fig. 4d).

In experiments to test the effects of PUFAs on the COX-2 promoter following NF- κ B site deletion, arachidonic acid failed to induce CAT expression in any of the three NF- κ B mutant constructs (Fig. 4e). Similar results were obtained with 4 β -PMA and prostaglandin F_{2 α} , and with dihomogammalinolenic, linoleic and conjugated linolenic acids (data not shown). As expected on the basis of the effect of MG132 observed in Fig. 4b, removal of the NF- κ B sites from pCATCOXprom reduced basal CAT expression by the constructs to below 1% of the control level.

DISCUSSION

Previous experiments showed that one pathway to induction of COX-2 in uterine stromal cells treated with arachidonic acid involves PPAR α (Sheldrick et al., 2007). However in previous experiments it was not possible to distinguish between an effect of arachidonic acid as a PPAR agonist and that involving activation of PKC and NF- κ B. Inhibitors were used to block either PKC or NF- κ B, but blocking PKC led to inhibition of the PPAR pathway (as expected based on the requirement for PPARs to be phosphorylated by PKC; Blanquart et al., 2004). Furthermore the PPAR α antagonist MK886 was ineffective in uterine stromal cells (Sheldrick et al., 2007). As a result it was difficult to identify PPAR-independent pathways. This difficulty has been avoided in the present experiments by using a reporter construct which is unresponsive to PPARs. As a PPAR-responsive reporter construct was not available (the PPRE not being identified to date in the bovine gene), endogenous COX-2 expression acted as a positive control.

The results reported here confirm the involvement of PPARs in COX-2 induction, as the synthetic PPAR agonists ciprofibrate, methyclofenapate, indomethacin and NS398, which induce the endogenous gene (Fig. 3c; Sheldrick et al., 2007), did not induce the PPRE-negative CAT construct. However the data revealed at least one PPAR-independent pathway for induction of COX-2 promoter function by PUFAs, as the COX-2 promoter construct used was induced by arachidonic acid and other unsaturated fatty acids (Fig. 3a and b). This was confirmed by the time course of CAT induction by PUFAs (occurring at 12 - 48 h; Fig. 3), which differed from that of the endogenous COX-2 gene observed by immunoblotting (which

peaked at 6 h). The delay in expression of CAT by PUFAs was not due to damage caused to the cells during electroporation, as expression of COX-2 from the endogenous gene was induced in electroporated cells at 6 h by indomethacin, which has previously been shown to be an effective PPAR agonist.

One potential PPAR-independent route to COX-2 induction involves PKC and NF- κ B (Scheme 1). All three PKC activators tested here: PUFAs (Shinomura et al., 1991; Khan et al., 1995), 4 β -PMA (Nishizuka, 1984) and prostaglandin F_{2 α} (Abayasekara et al., 1993) induced CAT expression, and each of these effects was blocked by the NF- κ B inhibitor, MG132. Furthermore the role of NF- κ B was confirmed by the deletion studies which showed that induction of the *COX-2* promoter by arachidonic acid, 4 β -PMA or prostaglandin F_{2 α} depended on the presence of an NF- κ B responsive element. These findings are consistent with pathways c, d, e and f in Scheme 1.

The effects of arachidonic acid and 4 β -PMA on CAT expression were also prevented by the I- κ B kinase inhibitor parthenolide (4 μ M; data not shown). Parthenolide maintains I- κ B in the non-phosphorylated state, and as a result inhibits translocation of NF- κ B to the nucleus (Bork et al., 1997). Its action is therefore similar to that of MG132, which reduces the rate of I- κ B degradation. However, in contrast to the effect of MG132, parthenolide increased basal CAT expression. Among the non-specific effects of sesquiterpene lactones such as parthenolide is the inhibition of 5-lipoxygenase (Capasso, 1986; Sumner et al., 1992). As lipoxygenase products down-regulate COX-2 (Ait-Said et al., 2003), and the lipoxygenase inhibitor nordihydroguaiaretic acid increases COX-2 levels in stromal cells (E.L.R. Sheldrick, unpublished work), an increase in CAT expression in response to parthenolide may reflect a side effect of this compound.

The NF- κ B inhibitor MG132 decreased CAT expression dramatically, and deletion of NF- κ B sites from pCATCOXprom had the same effect. These effects reflect the importance of NF- κ B in regulating *COX-2* promoter function. A reduction in basal *COX-2* promoter function also follows NF- κ B site mutation in human endometrial stromal cells (Tamura et al., 2002) and alveolar epithelial cancer cells (Huang et al., 2003). The reduced level of CAT in these experiments suggests that a small increase in reporter expression in response to arachidonic acid or 4 β -PMA would have been detectable, had there been an alternative activatory site. The

double mutant was less active than either of the single mutants, but was not induced significantly by PUFA.

Use of the PKC inhibitor RO318425 showed that although the effect of 4 β -PMA (Scheme 1, pathway e, f) was dependent on PKC, the effect of arachidonic acid was not. This allowed the effect of arachidonic acid to be dissociated from PKC, revealing a PKC-independent pathway to NF- κ B activation by arachidonic acid (pathway g in Scheme 1). The existence of a PKC-independent pathway is consistent with the additive effects of arachidonic acid and PKC activators (Fig. 4a). Potential intermediates in this pathway include reactive oxygen species, which are involved in the activation of NF- κ B by PUFAs with a time course consistent with that observed here (24 – 48 h; Mazière et al., 1999; Bécuwe et al., 2003). The effect of arachidonic acid was blocked by inhibition of NF- κ B (Fig. 4b), ruling out an arachidonic acid-stimulated pathway to COX-2 induction independent of both PKC and NF- κ B.

Certain isoforms of PKC are down-regulated following activation, leading to refractoriness (Standaert et al., 1993). In separate studies we have confirmed that this occurs in bovine endometrial stromal cells exposed to 4 β -PMA for 24 h, as shown by a reduction in phosphorylation by PKC of cyclic AMP phosphodiesterase (Cheng et al., 2007). PKC would therefore be expected to be down-regulated following activation by arachidonic acid. On this basis, if the PPAR-independent induction of CAT expression by arachidonic acid required PKC activation, the effect might be expected to be lost after 24 h. The fact that it was not (as the effects of PUFAs were only observed at or after 24 h; Fig. 3) at first sight confirms the suggestion that this effect is not exerted via PKC. However this conclusion is difficult to reconcile with the action of 4 β -PMA, which increased CAT expression at 48 h, the effect being blocked by RO318425 (Fig. 4c). One possible explanation is that the isoform of PKC responsible for CAT induction by 4 β -PMA was resistant to down-regulation by phorbol ester. There is a precedent for this in that PKC isoforms differ in the speed and extent of their down-regulation by phorbol esters in a cell-specific manner (Adams and Gullick, 1989; Standaert et al., 1993). There are therefore 3 reasons for concluding that PKC is not involved in the response to arachidonic acid: (i) the response is not blocked by RO318425; (ii) the responses to arachidonic acid and 4 β -PMA or prostaglandin F_{2 α} are additive, and (iii) the effect occurs at a time when PKC would be expected to be down-regulated. In the light of these findings we conclude that arachidonic acid activates COX-2 promoter function in the absence of a PPRE,

demonstrating the existence of a PPAR-independent route to COX-2 expression, and that NF- κ B (but not PKC) is involved in this response.

We thank Pat Fisher for isolating the endometrial stromal cells. This work was funded by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

REFERENCES

- Abayasekara, D.R.E., Jones, P.M., Persaud, S.J., Michael, A.E., Flint, A.P.F., 1993. Prostaglandin $F_{2\alpha}$ activates protein kinase C in human ovarian cells. *Mol. Cell. Endocrinol.* 91, 51-57.
- Adams, J.C., Gullick, W.J., 1989. Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines. *Biochem. J.* 257, 905-911.
- Ait-Said, F., Elalamy, I., Werts, C., Gomard, M.-T., Jacquemin, C., et al., 2003. Inhibition by eicosapentanoic acid of IL-1 β -induced PGHS-2 expression in human microvascular endothelial cells: involvement of lipoxygenase-derived metabolites and p38 MAPK pathway. *Biochim. Biophys. Acta* 1631, 77-84.
- Asselin, E., Drolet, P., Fortier, M.A., 1997. Cellular mechanisms involved during oxytocin-induced prostaglandin $F_{2\alpha}$ production in endometrial epithelial cells *in vitro*: role of cyclooxygenase-2. *Endocrinology* 138, 4798-4805.
- Bécuwe, P., Bianchi, A., Didelot, C., Barberi-Heyob, M., Dauça, M., 2003. Arachidonic acid activates a functional AP-1 and an inactive NF- κ B complex in human HepG2 hepatoma cells. *Free Radical Biol. Med.* 35, 636-647.
- Blanquart, C., Mansouri, R., Paumelle, R., Fruchart, J.-C., Staels, B., et al., 2004. The protein kinase C signaling pathway regulates a molecular switch between transactivation and transrepression activity of the peroxisome proliferator-activated receptor α . *Mol. Endocrinol.* 18, 1906-1918.
- Bork, P.M., Schmitz, M.L., Kuhnt, M., Escher, C., Heinrich, M., 1997. Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF- κ B. *FEBS Lett.* 402, 85-90.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

- Capasso, F., 1986. The effect of an aqueous extract of *Tanacetum parthenium* L. on arachidonic acid metabolism by rat peritoneal leucocytes. *J. Pharm. Pharmacol.* 38, 71-71.
- Cheng, Z., Sheldrick, E.L., Marshall, E., Wathes, D.C., Abayasekara, D.R.E., et al., 2007. Control of cyclic AMP concentration in bovine endometrial stromal cells by arachidonic acid. *Reproduction* 133, 1017-1026.
- Cooke, B.A., Dirami, L., Chaudry, L., Choi, M.S.K., Abayasekara, D.R.E., et al., 1991. Release of arachidonic acid and the effects of corticosteroids on steroidogenesis in rat testis Leydig cells. *J. Steroid Biochem. Mol. Biol.* 40, 465-471.
- Flint, A.P.F., Leat, W.M.F., Sheldrick, E.L., Stewart, H.J., 1986. Stimulation of phosphoinositide hydrolysis by oxytocin and the mechanism by which oxytocin controls prostaglandin synthesis in the ovine endometrium. *Biochem. J.* 237, 797-805.
- Flint, A.P.F., Sheldrick, E.L., Fisher, P.A., 2002. Ligand-independent activation of steroid receptors. *Dom. Anim. Endocrinol.* 23, 13-24.
- Huang, W.-C., Chen, J.-J., Inoue, H., Chen, C.-C., 2003. Tyrosine phosphorylation and I- κ B kinase α/β by protein kinase C-dependent c-Src activation is involved in TNF- α -induced cyclooxygenase-2 expression. *J. Immunol.* 170, 4767-4775.
- Ijpenberg, A., Jeannin, E., Wahli, W., Desvergne, B., 1997. Polarity and specific sequence requirement of peroxisome proliferator-activated receptor (PPAR)/RetinoidX receptor heterodimer binding to DNA. *J. Biol. Chem.* 272, 20108-20117.
- Khan, W.A., Blobe, G.C., Hannun, Y.A., 1995. Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. *Cell. Signal.* 7, 171-184.
- Lee, J.Y., Sohn, K.H., Rhee, S.H., Hwang, D., 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. *J. Biol. Chem.* 276, 16683-16689.

Liu, J., Antaya, M., Boerboom, D., Lussier, J.G., Silversides, D.W., et al., 1999. The delayed activation of the prostaglandin G/H synthase-2 promoter in bovine granulosa cells is associated with down-regulation of truncated upstream stimulatory factor-2. *J. Biol. Chem.* 274, 35037-35045.

MacLaren, L.A., Guzeloglu, A., Michel, F., Thatcher, W.W., 2006. Peroxisome proliferator-activated receptor (PPAR) expression in cultured bovine endometrial cells and response to omega-3 fatty acid, growth hormone and agonist stimulation in relation to series 2 prostaglandin production. *Dom. Anim. Endocrinol.* 30, 155-169.

Maloberti, P., Castilla, R., Castillo, F., Maciel, F.C., Mendez, C.F., et al., 2005. Silencing the expression of mitochondrial acyl-CoA thioesterase I and acyl-synthetase 4 inhibits hormone-induced steroidogenesis. *FEBS J.* 272, 1804-1814.

Mazière, C., Conte, M.-A., Degonville, J., Ali, D., Mazière, J.-C., 1999. Cellular enrichment with polyunsaturated fatty acids induces an oxidative stress and activates the transcription factors AP1 and NFκB. *Biochem. Biophys. Res. Comm.* 265, 116-122.

Meade, E.A., McIntyre, T.M., Zimmerman, G.A., Prescott, S.M., 1999. Peroxisome proliferators enhance cyclooxygenase-2 expression in epithelial cells. *J. Biol. Chem.* 274, 8328-8334.

Nishizuka, Y., 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature, Lond.* 308, 693-698.

Paik, J.H., Ju, J.H., Lee, J.Y., Boudreau, M.D., Hwang, D.H., 2000. Two opposing effects of non-steroidal anti-inflammatory drugs on the expression of the inducible cyclooxygenase. *J. Biol. Chem.* 275, 28173-28179.

Parent, J., Villeneuve, C., Fortier, M.A., 2003. Evaluation of the contribution of cyclooxygenase 1 and cyclooxygenase 2 to the production of PGE₂ and PGF_{2α} in epithelial cells from bovine endometrium. *Reproduction* 126, 539-547.

- Prigent-Tessier, A., Pageaux, J.-F., Fayard, J.-M., Lagarde, M., Laugier, C., et al., 1996. Arachidonic acid up-regulates and prostaglandin E₂ down-regulates the expression of pancreatic-type phospholipase A₂ and prostaglandin-endoperoxide synthase 2 in uterine stromal cells. *Eur. J. Biochem.* 241, 872-878.
- Robinson, R.S., Mann, G.E., Lamming, G.E., Wathes, D.C., 1999. The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. *J. Endocrinol.* 160, 21-33.
- Ronco, A.M., Moraga, P.F., Llanos, M.N., 2002. Arachidonic acid release from rat Leydig cells: the involvement of G protein, phospholipase A₂ and regulation of cAMP production. *J. Endocrinol.* 172, 95-104.
- Sheldrick, E.L.R., Derecka, K., Marshall, E., Chin, E.C., Hodges, L., et al., 2007. Peroxisome-proliferator-activated receptors and the control of levels of prostaglandin-endoperoxide synthase 2 by arachidonic acid in the bovine uterus. *Biochem. J.* 406, 175-183.
- Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K., Nishizuka, Y., 1991. Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: its possible implications. *Proc. Natl. Acad. Sciences U.S.A.* 88, 5149-5153.
- Standaert, M.L., Cooper, D.R., Hernandez, H., Arnold, T.P., Farese, R.V., 1993. Differential down-regulation of insulin-sensitive protein kinase-C isoforms by 12-*O*-tetradecanoylphorbol-13-acetate in rat adipocytes and BC3H-1 myocytes. *Endocrinology.* 132, 689-692.
- Sumner, H., Salan, U., Knight, D.W., Hoult, J.R.S., 1992. Inhibition of 5-lipoxygenase and cyclo-oxygenase in leukocytes by feverfew. *Biochem. Pharmacol.* 43, 2313-2320.
- Tamura, M., Sebastian, S., Yang, S., Gurates, B., Fang, Z., et al., 2002. Interleukin-1 β elevates cyclooxygenase-2 protein level and enzyme activity via increasing its mRNA stability in human endometrial stromal cells: an effect mediated by extracellularly regulated kinases 1 and 2. *J. Clin. Endocrinol. Metab.* 87, 3263-3273.

- 501
502 Wu, W.-T., Chi, K.-H., Ho, F.-M., Tsao, W.-C., Lin, W.-W., 2004. Proteasome inhibitors
503 up-regulate haem oxygenase-1 gene expression: requirement of p38 MAPK (mitogen-
504 activated protein kinase) activation but not of NF- κ B (nuclear factor κ B) inhibition.
505 Biochem. J. 379, 587-593.
- 506
507 Wu, Y.-L., Wiltbank, M.C., 2001. Transcriptional regulation of cyclooxygenase-2 gene in
508 ovine large luteal cells. Biol. Reprod. 65, 1565-1572.
- 509
510 Yan, X., Xiao, C.W., Sun, M., Tsang, B.K., Gibb, W., 2002. Nuclear factor kappa B
511 activation and regulation of cyclooxygenase type-2 expression in human amnion
512 mesenchymal cells by interleukin-1 β . Biol. Reprod. 66, 1667-1671.
- 513

Figure Legends

Figure 1. (a) Fragment of bovine *COX-2* gene promoter. Numbering as given in GenBank accession no. **AF031699** at left, from the transcription start site (TSS) at right. Throughout this paper, numbering is from the transcription start site. Block arrows mark transcription and translation start sites (indicated by **TSS** and **coding sequence** respectively). Thin arrows mark PCR primers used to produce the insert in pCATCOXprom. Half sites of native PPREs (after Ijpenberg et al., 1997) are underlined and highlighted. The almost perfect match to the native PPRE present in the human lipoprotein lipase promoter at position -109 – -93 of the bovine *COX-2* promoter sequence is highlighted (13 out of 17 nucleotides being identical). The NF- κ B sites I and II are in bold and underlined. (b) Mutations introduced in both NF- κ B sites (Lee et al., 2001).

Figure 2. CAT expression in stromal cells transfected with pCATCOXprom and treated with PPAR agonists. Cells transfected by electroporation were cultured for 36 h with or without the following PPAR agonists which have previously been shown to raise endogenous *COX-2* levels in stromal cells (Sheldrick et al., 2007): ciprofibrate (50 μ M), methylclofenapate (50 μ M), indomethacin (10 μ M), NS398 (10 μ M) and prostaglandin A₁ (3 μ M). The PPAR γ agonist pioglitazone (50 μ M) has previously been shown to be ineffective. Arachidonic acid, but none of the other PPAR α agonists, induced CAT expression.

Figure 3. Time course of induction of CAT expression by arachidonic acid and other PUFAs. (a) Stromal cells transfected with pCATCOXprom were cultured in the presence of arachidonic acid (50 μ M) for up to 48 h. Closed bars, CAT expression; open bars, endogenous *COX-2* determined by immunoblotting in non-transfected cells (data of Sheldrick et al., 2007). *COX-2* levels were raised at 3 – 12 h, whereas CAT expression was raised only after 12 h. Inset shows representative immunoblotting signals for *COX-2*. (b) Specificity of CAT induction by PUFAs. Stromal cells transfected with pCATCOXprom were cultured in the presence or absence of dihomogammalinolenic acid (DGLA; 50 μ M), conjugated linoleic acid (CLA; 50 μ M) or linoleic acid (LA; 2 μ M) for up to 48h. The concentration of linoleic acid tested, 2 μ M, was optimal in dose response experiments (data not shown). (c) Electroporated cells remained responsive to PPAR ligand. To test whether the failure of PPAR ligands to induce *COX-2* promoter function at 6 h (Figs. 2 and 3a) reflected damage to cells caused by electroporation,

cells were cultured with the PPAR ligand indomethacin with (Electroporated) and without (Control) electroporation. COX-2 levels were measured by immunoblotting after 6 h treatment. Indomethacin increased COX-2 levels both before and after electroporation, showing that expression from the endogenous *COX-2* gene at 6 h was not prevented by electroporation.

Figure 4. Induction of CAT expression by arachidonic acid, 4 β -PMA and prostaglandin F_{2 α} . Stromal cells transfected with pCATCOXprom (or related NF- κ B deletion mutants) were cultured with arachidonic acid (50 μ M) for 48 h with or without the PKC activators 4 β -PMA (2 μ M) and prostaglandin F_{2 α} (3 μ M), the PKC inhibitor RO318425 (500 μ M), the NF- κ B inhibitor MG132 (30 μ M) or the inactive phorbol ester 4 α -phorbol 12,13-didecanoate (2 μ M). (a) CAT expression increased in response to arachidonic acid, 4 β -PMA and prostaglandin F_{2 α} , and the effects of arachidonic acid and either 4 β -PMA or prostaglandin F_{2 α} were additive. 4 α -Phorbol 12,13-didecanoate (4 α -PDD) had no effect. (b) The effects of both arachidonic acid and 4 β -PMA were prevented by MG132. Note that CAT expression was decreased by MG132 added alone. (c) The effect of 4 β -PMA was prevented by the PKC inhibitor RO318425, but (d) the effect of arachidonic acid was not. (e) The response to arachidonic acid was blocked by deletion of either of the NF- κ B sites in the reporter plasmid pCATCOXprom. Del I, -472/-463 mutation; Del II, -454/-445 mutation; Del I/II, mutations at both -454/-445 and -472/-463. Basal CAT expression in mutation Del I/II was lower than for the other mutant constructs, but the effect of arachidonic acid was not statistically significant.

Scheme 1. Scheme illustrating possible pathways by which arachidonic acid (AA) induces COX-2 expression (or, in reporter experiments, expression of chloramphenicol acetyl transferase, CAT) in stromal cells. AA induces expression of the endogenous *COX-2* gene through activation of PPAR α (a) (Sheldrick et al., 2007). Protein kinase C (PKC) activates PPAR α through phosphorylation (b) (Blanquart et al., 2004). These pathways are shown as dotted lines because the COX-2 promoter construct used in the present experiments to express CAT lacked a peroxisome-proliferator-activated receptor response element, and did not respond to PPAR α activation. PKC may be activated directly by AA (c), indirectly by prostaglandin F_{2 α} synthesised from AA via the FP receptor and phospholipase C (d) or by the phorbol ester 4 β -PMA (e). PKC induces COX-2 via NF- κ B (f). The experiments reported here suggest a third pathway by which AA induces COX-2 via an unspecified intermediate X acting through NF- κ B (g). We have not attempted to identify isoforms of PKC or NF- κ B, or

580 intermediates between PKC and NF- κ B. After Sheldrick et al. (2007), which provided evidence
581 for pathways a – f, but not for pathway g.

582

Accepted Manuscript

Figure 1

(a)

241 ttttttgggg ggtggggga gggggtggca tttcaccttc agacaccctt **PCR F primer** → -1350
 301 **agacagaatc** aaacaaatgc aaaagatgat gaaattgaaa attgtcaaaa tcacccttcc -1290
 361 ttcatatcca ttccatccga attcctcatc aaattacctt ccaaaatgag cctagaaaaac -1230
 421 tggccctaataa aaaccagac aactgaaaag tgagtgtaat gttgtacttt aatcctttgt -1170
 481 cacaactcat tatcctagag aagtaggcag aaaacatgca cacaaccaa tctttaaaaa -1110
 541 tctacttcct tcaattggta tctcctgtga aaagctggcg accaaaacag tgaaaatcac -1050
 601 tgcagcagag aaagaaatgc cataaagcag atcttttggga cggtagcat ccttaaacat -990
 661 cccggtcacc tccgcttcac ttgttcattg gaggagagggt tacagcgggtg acccctcaat -930
 721 ctcagattga ctatttacag gataagtgtc gggtcagt tttggggga ggaatttacc -870
 781 tttatttcct ctctccaaga agcaacgtcg ggggtggggc atgaagaaca gtatttttta -810
 841 tggtgaaaac aacttagcta gaaaaagaac cttacaggta cgtatacact gactactgct -750
 901 attacagttc attcgggtcaa gtaaaaaaat aaaaaaatta aagtattaat aaaaaatatt -690
 961 aaagtattaa taaaaaatta aaaattaatg gatgtgcgta catctacacg aggttaagag -630
 1021 tagtactga aaccaggaca catctgaaat acatgacatg agtaaaacat gtcagccttt -570
 1081 cttggcccga ttctcccag tctctccaga ctgtgacttc ctcgccccc aaaagccgag -510

 1141 ggaccagacg tagggggacg ggggtggggg cgcgcgagg **NFκB I** **aattccctgc** gctccggaac -450
 1201 gtcccagccc cctgatcaat tccgggagag gaagcaagcc aggaggccgc cctcccacc -390
 1261 cgcgccctca cccagaaaat ctacctcgga cccctctctg gaagcctcg tcccaaccgc -330
 1321 gacagaagga aaggaatctg gcgcgaagcc ggccgagcg gcggagaaac cggccgaggg -270
 1381 gcgctggaag gtctgaactg gaagggggcg atcctctttg caccacacgt aggaagccaa -210
 1441 gcggcgactc cccaagtttc cgattttcta gtttgctag ctaaaaaaa aagaaagaaa -150
 1501 tcctacccca acccggtct tgcgcaattg ttttaagtag aagaggggga **aaagttttg** -90
 1561 aaggggggaa ggaaaggcg aaagaaacag tcatgccgtc acgtgggcta tttcacgcat -30

 1621 aaaaggaag **gcctctccgt** **tagcttcag** **ttgtcaaagg** actcgagtg aacgtcagag +31

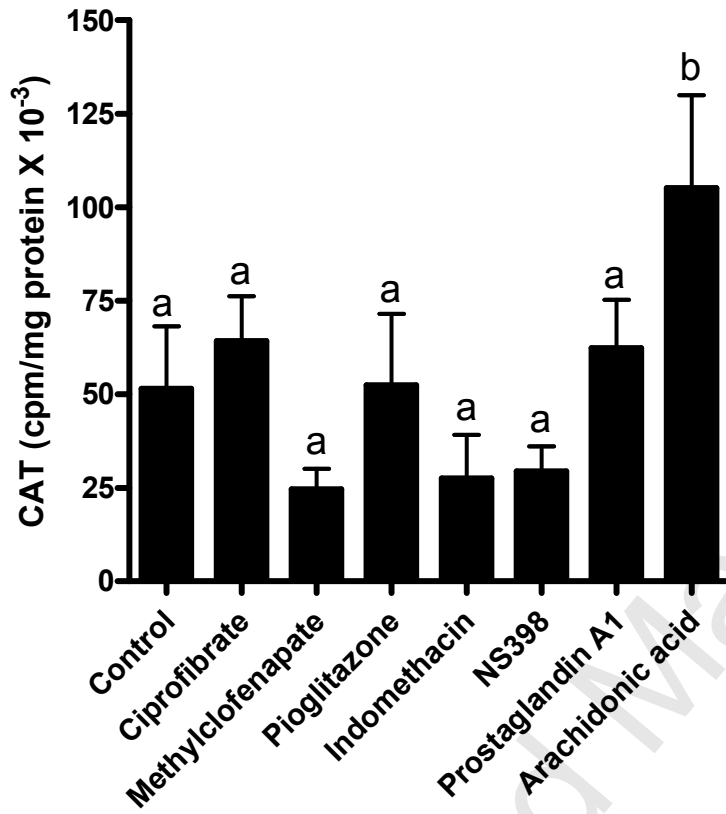
 1681 gacgccccgg aactccgcac cgccctctc cgccccgca gctccgatcc gcgcacctcc +91
 1741 acgcctccgc cggccccgcg ccccgccgc agccgag**atg** ctcgccccgg cctgctgct +151

PCR R primer ←
coding sequence

(b)

5'-NF-κB 3'-NF-κB
 cgcgcgaggggaattccctgc gctccggaacgtcccag
 ||||| || |||||
 cgcgcgag.ggccttccctgc gctccgg.ccgtcccag

Figure 2



66

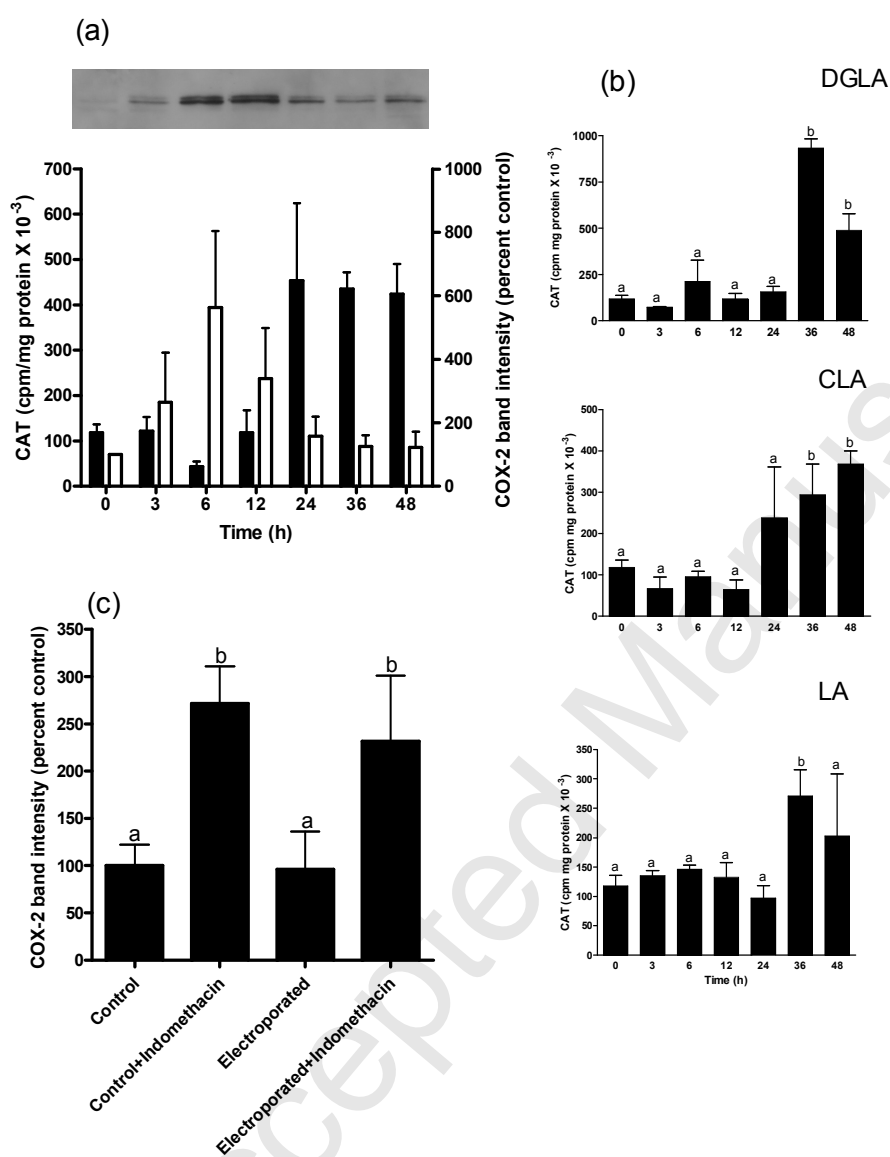
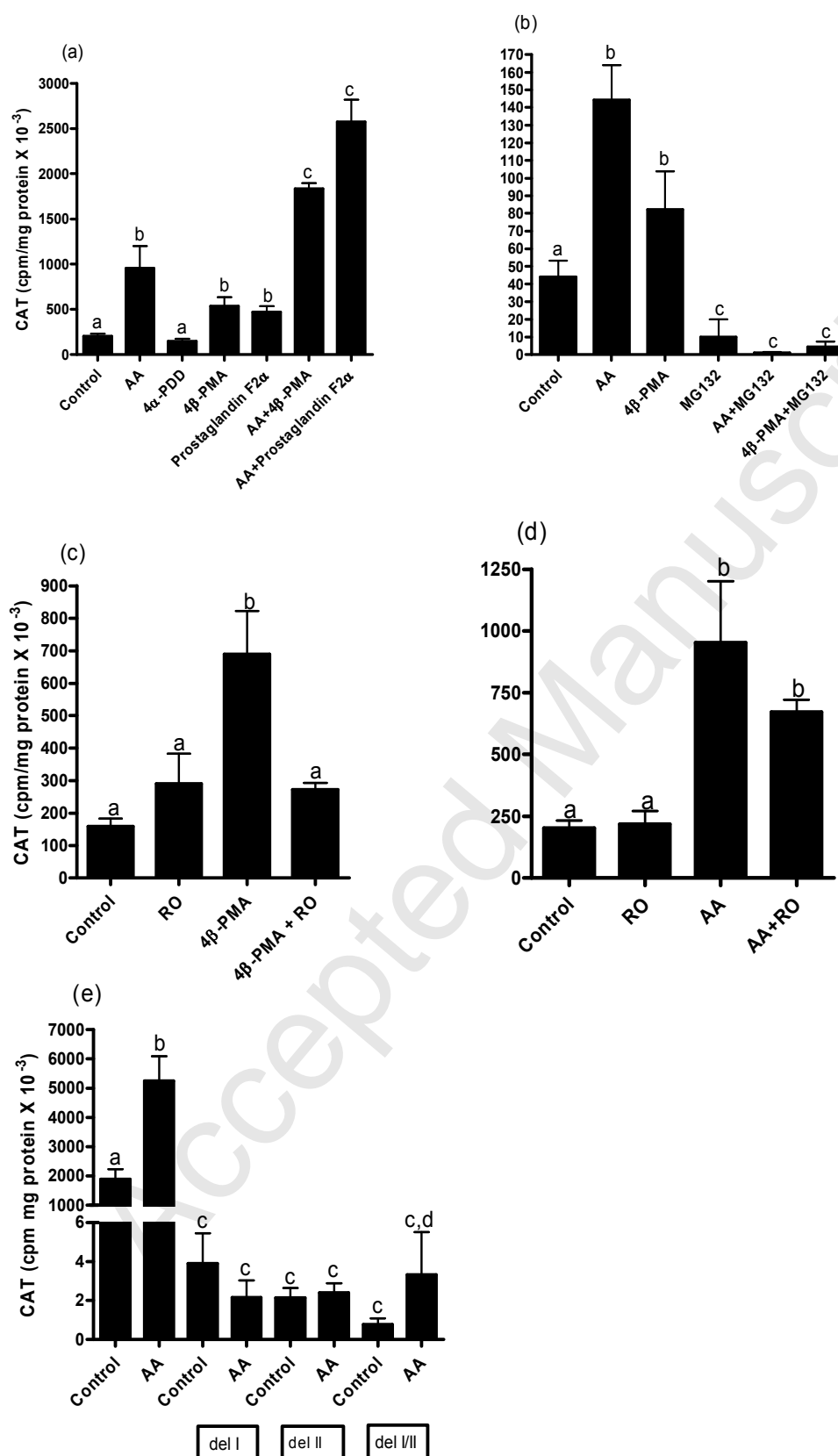
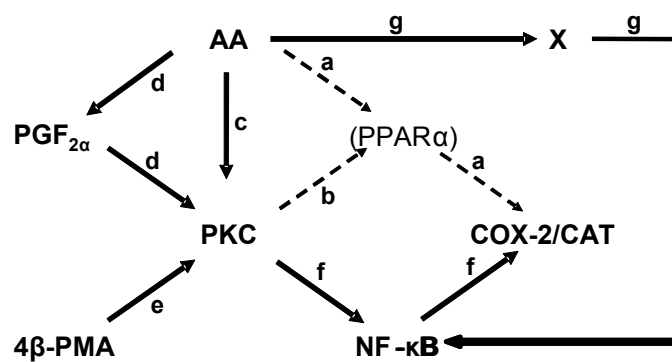


Figure 4



69

Scheme 1



70