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3 **A PPAR-independent pathway to PUFA-induced COX-2 expression**
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39 Keywords: Arachidonic acid; Protein kinase C; Peroxisome-proliferator-activated receptor;
40 Cyclooxygenase 2; (Bovine endometrium).

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44

45 Summary

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

58 **INTRODUCTION**

59
60 Various hormones interact with G-protein-linked cell surface receptors to activate
61 phospholipases and raise intracellular levels of polyunsaturated fatty acids (PUFAs). A good
62 example is the effect of oxytocin exerted via oxytocin receptors on the epithelium of the
63 uterine endometrium, which leads to activation of phospholipases C and A, releasing
64 arachidonic acid and other PUFAs esterified in phospholipids (Flint et al., 1986). PUFAs
65 produced in this way may then act as second messengers, both through activation of protein
66 kinases and via other metabolic products (Khan et al., 1995; Maloberti et al., 2005).

67
68 PUFAs may also be secreted by the cells in which they are generated, to act on neighbouring
69 cells as paracrine messengers (Cooke et al., 1991; Ronco et al., 2002). This has been suggested
70 to occur in the endometrium in relation to both activation of cyclic AMP phosphodiesterase
71 (Cheng et al., 2007) and induction of cyclooxygenase 2 (COX-2; also known as prostaglandin
72 endoperoxide synthase 2; Sheldrick et al., 2007). Evidence for an effect on COX-2 includes the
73 observations that PUFAs raise COX-2 levels in rat uterine stromal cells (Prigent-Tessier et al.,
74 1996) and in bovine endometrial epithelial cells (Parent et al., 2003) through increased *COX-2*
75 gene expression and increased levels of COX-2 transcripts (Asselin et al., 1997; Meade et al.,
76 1999). Furthermore the oxytocin receptor is expressed in the bovine endometrial epithelium at
77 a time when it is not expressed in the stroma (Robinson et al., 1999). Arachidonic acid has
78 therefore been proposed to act as a paracrine signal released during the oestrous cycle by
79 epithelial cells to induce COX-2 in the stroma (Sheldrick et al., 2007). The experiments
80 described here were carried out to investigate the pathways by which PUFAs induce *COX-2*
81 gene expression in endometrial stromal cells.

82
83 Previous experiments on the mechanism by which arachidonic acid raises COX-2 levels
84 suggest that peroxisome-proliferator-activated receptors (PPARs) mediate PUFA-induced
85 COX-2 expression in the bovine endometrium (MacLaren et al., 2006; Sheldrick et al., 2007).
86 The isoform PPAR α appears to be involved in bovine endometrial stromal cells. PPAR α is
87 activated directly by a range of PUFAs, including arachidonic acid. However, PPAR α function
88 is dependent on phosphorylation by protein kinase C (PKC; Blanquart et al., 2004), which is
89 also activated by PUFAs (Shinomura et al., 1991; Khan et al., 1995). Furthermore, in some
90 cells PKC induces COX-2 through a second pathway, involving NF- κ B (Paik et al., 2000; Wu

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

93
94 To distinguish between PPAR-dependent and -independent pathways we have used a reporter
95 construct under the control of a naturally-occurring sequence derived from the bovine *COX-2*
96 promoter. This construct, which includes most of the important transcription regulators present
97 in the *COX-2* promoter but lacks an identifiable peroxisome-proliferator response element
98 (PPRE), allows the study of pathways to *COX-2* induction in which PPARs are not involved.
99 Furthermore we have investigated the role of NF- κ B in the function of the promoter by
100 sequential removal of the NF- κ B sites from this construct.

101

102 **MATERIALS AND METHODS**

103

104 **Materials**

105 All PUFAs; synthetic PPAR agonists; the NSAIDs, indomethacin and NS398; prostaglandin
106 A₁ (PGA₁); the phorbol ester 4 β -PMA and its inactive analogue 4 α -phorbol 12,13-didecanoate
107 (4 α -PDD); the protein kinase C inhibitor, RO 318425, and the NF- κ B inhibitor, MG132 were
108 obtained from either Sigma (Poole, UK) or Calbiochem (Nottingham, UK). Conjugated
109 linoleic acid (Sigma catalogue number O 5507) was a mixture of cis- and trans-9,11- and -
110 10,12-octadecadienoic acids. The PUFAs, indomethacin and prostaglandins were dissolved in
111 ethanol; all other compounds were added to culture medium in DMSO. Vehicle controls were
112 used as appropriate. PUFAs were stored in ethanol under N₂ at -20° C in darkness.

113

114 **Cell culture**

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

119

120 **COX-2 reporter plasmids**

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

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

134

135 **Transient transfections**

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

150

151 **Chloramphenicol acetyl transferase assay**

152 Chloramphenicol acetyl transferase (CAT) was measured in cell lysates by a 2 phase method.
153 Pre-cleared cell lysates (30 μ l) were incubated at 37° C with 10 μ l 5 mM chloramphenicol in
154 250 mM Tris containing 5 mM EDTA, pH 7.8 in 96-well plates. After 5 min, 10 μ l 75 μ M HCl
155 containing 1.5 nmol [3 H]acetylcoenzyme A (Amersham, Little Chalfont, UK) was added to
156 each tube. The plates were incubated at 37° C for 3 h. The reaction mixture (48 μ l) was

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

161

162 **Immunoblotting**

163 Cells were lysed and extracted for immunoblotting as described previously (Sheldrick et al.,
164 2007). For immunoblotting, boiled lysates (10 µg protein) were subjected to electrophoresis on
165 10% acrylamide gels (5% stacking gels) before electroblotting onto Protran BA83 (Schleicher
166 & Schuell, Anderman and Company Kingston-upon-Thames, Surrey, UK) membranes in 25
167 mM Tris pH 8.3 containing 148 mM glycine and 20% (v/v) methanol. For detection of COX-2
168 bands (at molecular mass = 72 kDa), membranes were probed with COX-2 antibody (C-20; SC
169 1745, Santa Cruz Biotechnology, obtained through Autogen Bioclear, Calne, UK; 1:250
170 dilution in a solution containing 1% (w/v) Marvel milk powder and 0.5% (w/v) Tween 20 in
171 phosphate buffered saline). Second antibody was donkey anti-goat IgG-horseradish peroxidase
172 (SC 2020; Santa Cruz Biotechnology; 1:11 000 dilution in 3% (w/v) Marvel, 0.5% (w/v)
173 Tween 20 in phosphate buffered saline), and visualization was by enhanced
174 chemiluminescence (Amersham) using Kodak BioMax Light film. Colour markers (molecular
175 masses 29 000 – 205 000; Sigma) were used to identify molecular masses of proteins. Band
176 intensities were quantified using Kodak 1D digital image analysis software.

177

178 **Protein assay**

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

182

183 **Experimental design and analysis**

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

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

197

198 **RESULTS**

199

200 **Structure of the COX-2 promoter**

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

218

219 **pCATCOXprom was not responsive to PPAR activation**

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

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

229

230 **Time course of effects of PUFAs on CAT expression**

231 Further evidence that the effect of arachidonic acid (Fig. 2) was not due to activation of a
232 PPAR was obtained from time course studies. At concentrations above 10 μ M, arachidonic
233 acid increased CAT expression between 12 and 24 h after addition (Fig. 3a). Similar responses
234 were observed with n-6 PUFAs other than arachidonic acid (Fig. 3b), although these were
235 delayed until 24 – 36 h. In contrast, induction of the endogenous *COX-2* gene (as indicated by
236 immunoblotting) peaked at 6 h after exposure to agonist (Fig. 3a; data of Sheldrick et al.,
237 2007). The lag before expression of CAT relative to induction of the endogenous COX-2
238 protein did not reflect the time taken by the cells to recover from electroporation, as the PPAR
239 agonist indomethacin raised COX-2 levels within 6 h in cells that had been electroporated (Fig.
240 3c).

241

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

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

250

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

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

261
262 In experiments to test the effects of PUFAs on the COX-2 promoter following NF- κ B site
263 deletion, arachidonic acid failed to induce CAT expression in any of the three NF- κ B mutant
264 constructs (Fig. 4e). Similar results were obtained with 4 β -PMA and prostaglandin F_{2 α} , and
265 with dihomo γ -linolenic, linoleic and conjugated linolenic acids (data not shown). As
266 expected on the basis of the effect of MG132 observed in Fig. 4b, removal of the NF- κ B sites
267 from pCATCOXprom reduced basal CAT expression by the constructs to below 1% of the
268 control level.

269
270 **DISCUSSION**

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

283
284 The results reported here confirm the involvement of PPARs in COX-2 induction, as the
285 synthetic PPAR agonists ciprofibrate, methylclofenapate, indomethacin and NS398, which
286 induce the endogenous gene (Fig. 3c; Sheldrick et al., 2007), did not induce the PPRE-negative
287 CAT construct. However the data revealed at least one PPAR-independent pathway for
288 induction of COX-2 promoter function by PUFAs, as the COX-2 promoter construct used was
289 induced by arachidonic acid and other unsaturated fatty acids (Fig. 3a and b). This was
290 confirmed by the time course of CAT induction by PUFAs (occurring at 12 - 48 h; Fig. 3),
291 which differed from that of the endogenous COX-2 gene observed by immunoblotting (which

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

296
297 One potential PPAR-independent route to COX-2 induction involves PKC and NF- κ B (Scheme
298 1). All three PKC activators tested here: PUFAs (Shinomura et al., 1991; Khan et al., 1995),
299 4 β -PMA (Nishizuka, 1984) and prostaglandin F_{2 α} (Abayasekara et al., 1993) induced CAT
300 expression, and each of these effects was blocked by the NF- κ B inhibitor, MG132.
301 Furthermore the role of NF- κ B was confirmed by the deletion studies which showed that
302 induction of the *COX-2* promoter by arachidonic acid, 4 β -PMA or prostaglandin F_{2 α} depended
303 on the presence of an NF- κ B responsive element. These findings are consistent with pathways
304 c, d, e and f in Scheme 1.

305
306 The effects of arachidonic acid and 4 β -PMA on CAT expression were also prevented by the I-
307 κ B kinase inhibitor parthenolide (4 μ M; data not shown). Parthenolide maintains I- κ B in the
308 non-phosphorylated state, and as a result inhibits translocation of NF- κ B to the nucleus (Bork
309 et al., 1997). Its action is therefore similar to that of MG132, which reduces the rate of I- κ B
310 degradation. However, in contrast to the effect of MG132, parthenolide increased basal CAT
311 expression. Among the non-specific effects of sesquiterpene lactones such as parthenolide is
312 the inhibition of 5-lipoxygenase (Capasso, 1986; Sumner et al., 1992). As lipoxygenase
313 products down-regulate COX-2 (Ait-Said et al., 2003), and the lipoxygenase inhibitor
314 nordihydroguaiaretic acid increases COX-2 levels in stromal cells (E.L.R. Sheldrick,
315 unpublished work), an increase in CAT expression in response to parthenolide may reflect a
316 side effect of this compound.

317
318 The NF- κ B inhibitor MG132 decreased CAT expression dramatically, and deletion of NF- κ B
319 sites from pCATCOXprom had the same effect. These effects reflect the importance of NF- κ B
320 in regulating *COX-2* promoter function. A reduction in basal *COX-2* promoter function also
321 follows NF- κ B site mutation in human endometrial stromal cells (Tamura et al., 2002) and
322 alveolar epithelial cancer cells (Huang et al., 2003). The reduced level of CAT in these
323 experiments suggests that a small increase in reporter expression in response to arachidonic
324 acid or 4 β -PMA would have been detectable, had there been an alternative activatory site. The

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

327

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

338

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

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

360

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362

363

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514 **Figure Legends**

515

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

526

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

534

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

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

551
552 Figure 4. Induction of CAT expression by arachidonic acid, 4 β -PMA and prostaglandin F_{2 α} .
553 Stromal cells transfected with pCATCOXprom (or related NF- κ B deletion mutants) were
554 cultured with arachidonic acid (50 μ M) for 48 h with or without the PKC activators 4 β -PMA (2
555 μ M) and prostaglandin F_{2 α} (3 μ M), the PKC inhibitor RO318425 (500 μ M), the NF- κ B
556 inhibitor MG132 (30 μ M) or the inactive phorbol ester 4 α -phorbol 12,13-didecanoate (2 μ M).
557 (a) CAT expression increased in response to arachidonic acid, 4 β -PMA and prostaglandin F_{2 α} ,
558 and the effects of arachidonic acid and either 4 β -PMA or prostaglandin F_{2 α} were additive. 4 α -
559 Phorbol 12,13-didecanoate (4 α -PDD) had no effect. (b) The effects of both arachidonic acid
560 and 4 β -PMA were prevented by MG132. Note that CAT expression was decreased by MG132
561 added alone. (c) The effect of 4 β -PMA was prevented by the PKC inhibitor RO318425, but (d)
562 the effect of arachidonic acid was not. (e) The response to arachidonic acid was blocked by
563 deletion of either of the NF- κ B sites in the reporter plasmid pCATCOXprom. Del I, -472/-463
564 mutation; Del II, -454/-445 mutation; Del I/II, mutations at both -454/-445 and -472/-463.
565 Basal CAT expression in mutation Del I/II was lower than for the other mutant constructs, but
566 the effect of arachidonic acid was not statistically significant.

567
568 Scheme 1. Scheme illustrating possible pathways by which arachidonic acid (AA) induces
569 COX-2 expression (or, in reporter experiments, expression of chloramphenicol acetyl
570 transferase, CAT) in stromal cells. AA induces expression of the endogenous *COX-2* gene
571 through activation of PPAR α (a) (Sheldrick et al., 2007). Protein kinase C (PKC) activates
572 PPAR α through phosphorylation (b) (Blanquart et al., 2004). These pathways are shown as
573 dotted lines because the COX-2 promoter construct used in the present experiments to express
574 CAT lacked a peroxisome-proliferator-activated receptor response element, and did not
575 respond to PPAR α activation. PKC may be activated directly by AA (c), indirectly by
576 prostaglandin F_{2 α} synthesised from AA via the FP receptor and phospholipase C (d) or by the
577 phorbol ester 4 β -PMA (e). PKC induces COX-2 via NF- κ B (f). The experiments reported here
578 suggest a third pathway by which AA induces COX-2 via an unspecified intermediate X acting
579 through NF- κ B (g). We have not attempted to identify isoforms of PKC or NF- κ B, or

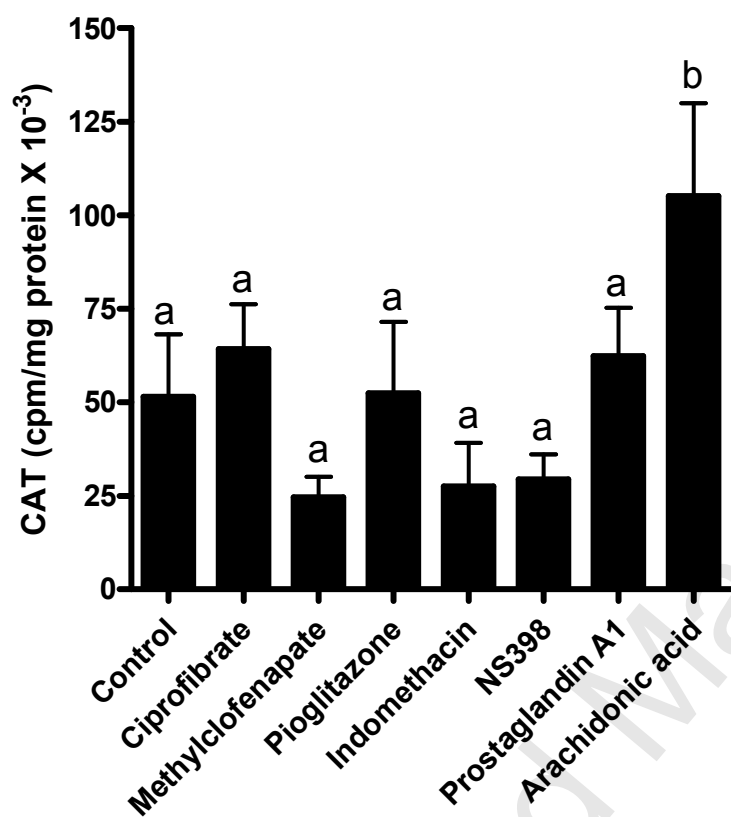
580 intermediates between PKC and NF- κ B. After Sheldrick et al. (2007), which provided evidence
581 for pathways a – f, but not for pathway g.

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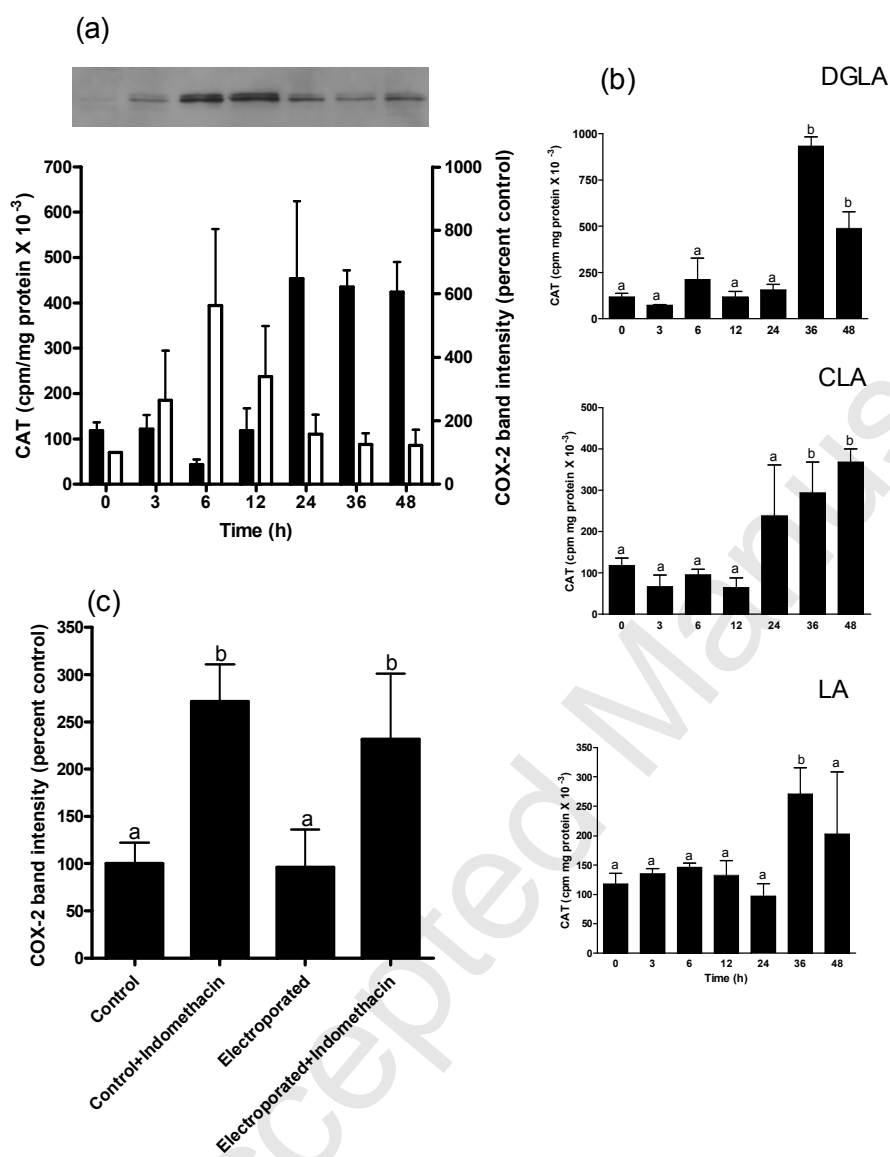
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Figure 2



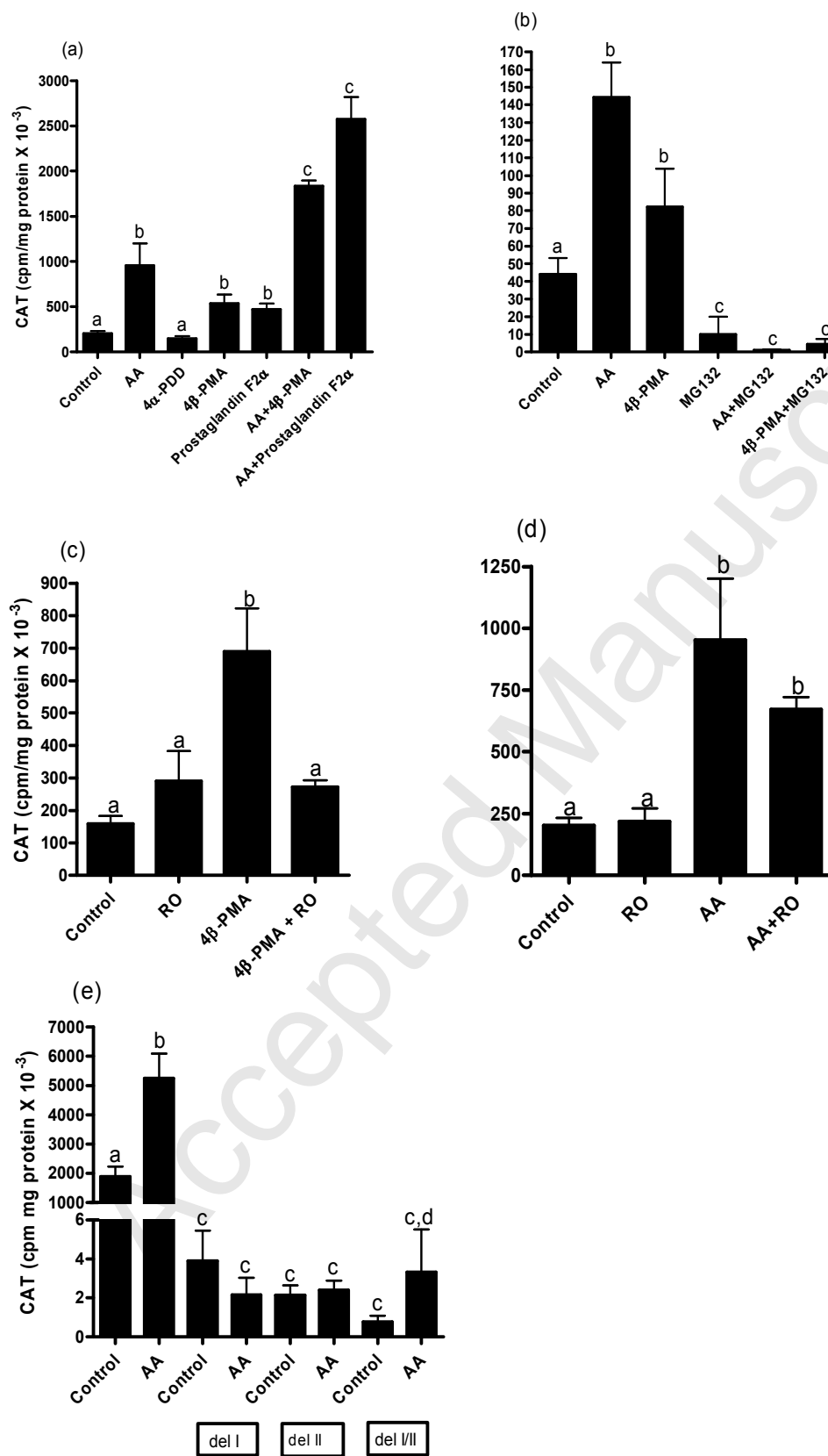
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Figure 3

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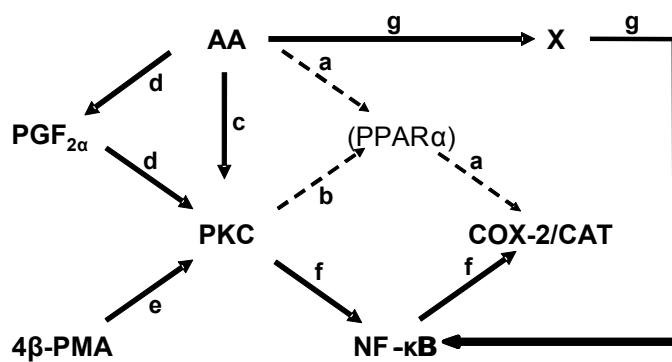
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Figure 4

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Scheme 1



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