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Allosteric receptor modulation of FFA2R turns natural agonists into potent

activators of the superoxide generating neutrophil NADPH-oxidase

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22 Key words: short chain free fatty acid receptor, FFA2R, GPCR, allosteric modulation,

23 priming, NADPH-oxidase, neutrophil, inflammation

24

25 **Abbreviations**

26 BSA = bovine serum albumin; CATB = the FFA2R antagonist 3-benzyl-4-(cyclopropyl-(4-

27 (2,5-dichlorophenyl) thiazol-2-yl)amino)-4-oxobutanoic acid]; CL = chemiluminescence;

28 Cmp1 = the FFA2R agonist 3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl) thiazol-2-

29 yl)amino)-4-oxobutanoic acid]; Cmp58 = the allosteric modulator (S)-2-(4-chlorophenyl)-3,3-

30 dimethyl-N-(5-phenylthiazol-2-yl)butanamide; CR3 = complement receptor 3; DAMP =

31 danger associated molecular pattern; FFA2R = free fatty acid receptor 2; FFA3R = free fatty

32 acid receptor 3; fMet = formyl-methionyl; fMLF = formyl-Met-Ley-Phe; FPR = formyl peptide

33 receptor; GPCR = G-protein coupled receptor; GPCR41 = free fatty acid receptor 3/FFA3R;

34 GPR43 = free fatty acid receptor 2/FFA2R; GPR84 = G-protein copled receptor 84; HRP =

35 horse radish peroxidase; IP_3 = inositol-1,4,5-tris-phosphate; KRG = Krebs-Ringer Glucose

36 phosphate buffer; MCPM = mega-counts per minute; O_2^- = superoxide anion; PAMP = pattern

37 associated molecular pattern; PLC; phospholipase C; PIP_2 = phosphatidyl-inositol-4,5-bis-

38 phosphate; PPR = pattern recognition receptors; SCFA = short chain fatty acid; TNF-

39 α = Tumor necrosis-α; WKYMVM = Trp-Lys-Tyr-Met-Val-L-Met; YM-254890 = a G-

40 proteinαq inhibitor; 4-CMTB = the allosteric agonist/modulator 4-Chloro-α-(1-methylethyl)-

41 N-2-thiazolyl-benzeneacetamide

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Abstract

Acetate, agonist for the free fatty acid receptor 2 (FFA2R/GPR43), triggers an increase in the cytosolic concentration of free Ca^{2+} in neutrophils without any assembly of the superoxide generating NADPH-oxidase. We show that the phenylacetamide compound 58 (Cmp58; (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl)butanamide, lacking a direct activating effect on neutrophils, acts as a positive allosteric FFA2R modulator that turns acetate into a potent activating agonist that triggers an assembly of the NADPH-oxidase. The NADPH-oxidase activity could be further increased in neutrophils treated with the pro-inflammatory cytokine TNF. Many neutrophil chemoattractant receptors are stored in secretory organelles but no FFA2R mobilization was induced in neutrophils treated with TNF. The receptor selectivity was demonstrated through the inhibition of the neutrophil response induced by the combined action of acetate and Cmp58 by the FFA2R antagonist CATPB. Allosteric modulators that positively co-operate with natural FFA2R agonists and prime neutrophils in their response to such agonists, may serve as good tools for further unraveling the physiological functions of the FFA2R and its involvement in various diseases. In this study, allosteric modulation of FFA2R is introduced as a novel receptor selective mechanism to prime neutrophils to produce increased amounts of reactive oxygen species.

Words: 193

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31   danger associated molecular pattern; FFA2R = free fatty acid receptor 2; FFA3R = free fatty

32   acid receptor 3; fMet = formyl-methionyl; fMLF = formyl-Met-Ley-Phe; FPR = formyl peptide

33   receptor; GPCR = G-protein coupled receptor; GPCR41 = free fatty acid receptor 3/FFA3R;

34   GPR43 = free fatty acid receptor 2/FFA2R; GPR84 = G-protein copled receptor 84; HRP =

35   horse radish peroxidase; IP_3 = inositol-1,4,5-tris-phosphate; KRG = Krebs-Ringer Glucose

36   phosphate buffer; MCPM = mega-counts per minute; O_2^- = superoxide anion; PAMP = pattern

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39   α = Tumor necrosis-α; WKYMVM = Trp-Lys-Tyr-Met-Val-L-Met; YM-254890 = a G-

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41   N-2-thiazolyl-benzeneacetamide

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Introduction

An inflammatory response is normally initiated, maintained, and sometimes modulated by microbial or host derived pathogen- or danger-associated molecular patterns (PAMPs and DAMPs) (1-3). It is clear that these DAMPs/PAMPs together participate in a multistep hierarchy of directional cues that regulate neutrophil function at inflammatory sites (4, 5). The importance of microbial products in regulating homeostasis and inflammatory responses in the gut is well known, and the number/amount of more or less well characterized and possibly also unknown metabolites released from growing bacteria that should be dealt with is very large. Peptides containing a formylated methionine (fMet) in their N-terminus constitute one such group of microbial derived "molecular pattern" which are recognized by cells of our innate immune system with the rational being that bacterial protein synthesis starts with an fMet (6). Short chain fatty acids (SCFA), produced through microbial fermentation of fiber-rich diets, are also of specific interest in relation to inflammatory diseases associated with the gut microbiota (7, 8). Products of bacterial metabolism such as formyl peptides and short chain fatty acids (SCFAs) exert their functions in relation to cells in our innate immune system through their binding to pattern recognition receptors (PRRs); formylated peptides being recognized by the formyl peptide receptors (FPRs; FPR1 and FPR2) (9-11)) whereas the SCFAs are recognized by two until fairly recently orphan receptors, FFA2R and FFA3R (earlier known as GPR43 and GPR41, respectively; (12, 13)). The FPRs as well as the FFARs belong to the family of G-protein coupled receptors (GPCRs (14)).

The SCFAs produced by bacteria can reach very high concentrations in the gut (15, 16), and they are subsequently transported over the gut epithelium to be released in the bloodstream where they may reach millimolar concentration levels. The SCFA receptor FFA3R (GPR41) has been shown not to be expressed in neutrophils whereas FFA2R is highly expressed in these cells (17), and published data suggest that the receptor has important roles in orchestration of

112   compound 58 (Cmp58; (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-

113   yl)butanamide) is one of them and it was recently classified as an allosteric FFA2R

114   agonist/modulator, when shown to change the efficacy of the response induced by acetate as

115   measured in a forskolin-induced cAMP assay system (22). In this study, we have investigated

116   the effects of this allosteric FFA2R agonist/modulator on neutrophils. Our data show that

117   Cmp58 functions as a positive allosteric modulator in these cells by turning the natural FFA2R

118   agonist acetate into a potent activator of the neutrophil NADPH-oxidase. A novel receptor

119   selective neutrophil priming mechanism is, thus, disclosed.

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Material and Methods

Chemicals

The peptides fMLF and WKYMVM were synthesized and HPLC-purified by TAG

Copenhagen A/S (Copenhagen, Denmark). Isoluminol, TNF-α, the FFA3R agonist

AR420626, PMA (phorbol 12-myristate 13-acetate), propionic acid, and pertussis toxin (PTX)

were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Cyclosporin H was

a kind gift provided by Novartis Pharma (Basel, Switzerland). Dextran and Ficoll-Paque were

obtained from GE-Healthcare Bio-Science (Uppsala, Sweden). Fura 2-AM was from

Molecular Probes/Thermo Fisher Scientific (Waltham, MA, USA), and horseradish

peroxidase (HRP) was obtained from Boehringer Mannheim (Mannheim, Germany). The

phenylacetamide compound (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-

yl)butanamide (PA;Cmp58) was obtained from Calbiochem-Merck Millipore (Billerica,USA;

(22)) and 4-chloro-α-(1-methylethyl)-N-2-thiazolyl-benzeneacetamide (4-CMTB) was from

TOCRIS (Bristol, UK).

Peptides were dissolved in DMSO and stored at -80ºC until use. Subsequent dilutions of all

peptides and other reagents were made in Krebs-Ringer Glucose phosphate buffer (KRG; 120

mM NaCl, 4.9 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose,

and 1 mM $CaCl_2$ in dH_2O, pH 7.3). Ammonium acetate (NH_4OAc) was from Merck (Germany),

and the Gαq inhibitor YM-254890 was purchased from Wako Chemicals (Neuss, Germany).

The phycoerythrine (PE) conjugated antibodies against CD11b were from Becton Dickinson

(New Jersey,USA) and against FFA2R/GPR43 were from Bios (Woburn, USA). The FFA2R

agonist Cmp1 (3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-

oxobutanoic acid and the antagonist CATPB ((S)-3-(2-(3-chlorophenyl)acetamido)-4-(4-

(trifluoromethyl)-phenyl) butanoic acid) synthesized as described previously (20, 31, 32), were

generous gifts from Trond Ulven (Odense university, Denmark).

149 **Isolation of neutrophils from human blood**

150 Neutrophils were isolated from buffy coats from healthy blood donors by dextran sedimentation

151 and Ficoll-Paque gradient centrifugation as described by Bøyum (33). Remaining erythrocytes

152 were removed by hypotonic lysis and the neutrophils were washed and resuspended in KRG

153 and stored on ice until use.

154 **Measuring NADPH-oxidase activity**

155 Isoluminol-enhanced chemiluminescence (CL) technique was used to measure superoxide

156 production, the precursor of production of reactive oxygen species (ROS), by the NADPH-

157 oxidase activity as described (34, 35). In short, the CL measurements were performed in a six-

158 channel Biolumat LB 9505 (Berthold Co., Wildbad, Germany), using disposable 4-ml

159 polypropylene tubes and a 900 μl reaction mixture containing 10^5 neutrophils, isoluminol (2x10$^-$

160 ^{5} M) and HRP (4 Units/mL). The tubes were equilibrated for 5 min at 37°C, before addition of

161 agonist (100 μl) and the light emission was recorded continuously over time. In experiments

162 where the effects of receptor specific antagonists were determined, these were added to the

163 reaction mixture 1-5 min before stimulation with control neutrophils incubated under the same

164 condition but in the absence of antagonist run in parallel for comparison. To determine the

165 involvement of Gαi, neutrophils (1x10^6/ml) in KRG were incubated without and with PTX

166 (500 ng/ml final concentration) at 37°C for different periods of time and the neutrophil

167 superoxide production was then determined.

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169 **Calcium mobilization**

170 Neutrophils at a density of 1–3x10^6 cells/ml were washed with Ca^{2+}-free KRG and centrifuged

171 at 220xg. The cell pellets were re-suspended at a density of 2x10^7 cells/ml in KRG containing

172 0.1% BSA, and loaded with 2 μM FURA 2-AM for 30 minutes at room temperature. The cells

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173   were then washed once with KRG and resuspended in the same buffer at a density of

174    $2x10^7$cells/ml. Calcium measurements were carried out in a Perkin Elmer fluorescence

175   spectrophotometer (LC50), with excitation wavelengths of 340 nm and 380 nm, an emission

176   wavelength of 509 nm, and slit widths of 5 nm and 10 nm, respectively. The transient rise in

177   intracellular calcium is presented as the ratio of fluorescence intensities (340 nm / 380 nm)

178   detected.

179   **Cell-surface receptor expression**

180   The level of surface expression of the integrin CR3 (CD11b) and FFA2R was determined by

181   the use of PE-conjugated antibodies against CD11b and FFA2R, respectively. Briefly,

182   neutrophils (5×10^6/mL) in KRG were incubated at 37°C in the absence or presence of TNF-α

183   (10 ng/mL; 20 min) before addition of antibodies and incubation on ice for 30 min in darkness.

184   Thereafter, cells were washed, to remove excess unbound antibodies, resuspended in KRG and

185   kept on ice in darkness until the amount of bound fluorescence was determined by flow

186   cytometry using an Accuri C6 flow cytometer (Becton Dickinson Sparks, MD, USA).

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190 **Results**

191 **Allosteric modulation of FFA2R lowers the concentration of acetate required to induce** a

192 **transient rise in the concentration of intracellular Ca^{2+} in neutrophils**

193 The receptor for acetate, FFA2R, belongs to the family of G-protein coupled receptors

194 (GPCRs). A very early signaling route in neutrophils, generated downstream of many agonist

195 activated GPCRs, is an increase in the cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_i$) (36),

196 triggered through the Giβ/γ-complex or the Gqα-subunit mediated PLC activation and cleavage

197 of PIP_2 (phosphatidyl-inositol-4,5-bis-phosphate) giving rise to inositol-1,4,5-tris-phosphate

198 (IP_3) and a transient Ca^{2+} rise. Accordingly, the FFA2R agonist acetate induced a concentration-

199 dependent transient increase in $[Ca^{2+}]_i$ when interacting with neutrophils (Fig 1 upper panel and

200 (23)). No such direct activating effect was induced by the phenylacetamide compound58

201 (Cmp58) or another earlier described allosteric FFA2R agonist, 4-CMTB (29), investigated in

202 concentrations up to 5μM (shown for Cmp58 in Fig 1 middle panel including also the response

203 induced by the FPR1 agonist fMLF for comparison). These compounds specifically recognizes

204 an allosteric FFA2R site and modulates receptor function (22, 29). The suggested positively

205 modulatory effect mediated by Cmp58 was clearly evident by comparing the neutrophil

206 response when stimulated with acetate alone (Fig 1 upper panel) and in the presence of Cmp58

207 (Fig 1 lower panel). At concentration where no rise in $[Ca^{2+}]_i$ could be detected with acetate

208 alone, neutrophils responded well when first incubated with Cmp58 (Fig 1 lower panel). In

209 summary, Cmp58 lowered the thresh-hold for the acetate-induced response as illustrated by the

210 fact that concentrations of the natural FFA2R agonist acetate that were non-activating in naïve

211 neutrophils induced a transient rise in the cytosolic concentration of free Ca^{2+} in cells pre-treated

212 with Cmp58 (Fig 1).

213 **Allosteric modulation of FFA2R turns acetate to a highly efficient activator of the**

214 **neutrophil NADPH-oxidase**

215 In addition to the triggering of the PLC-PIP$_2$-IP$_3$ signaling route that gives rise to a transient

216 increase in $[Ca^{2+}]_i$, many GPCR agonists also activate the NADPH-oxidase, an electron

217 transporting enzyme system in neutrophils that generates superoxide anions (O_2^-; (37)). In

218 agreement with the inability to mobilize Ca^{2+} (Fig 1 middle panel), Cmp58 had no direct

219 neutrophil activating effects on the oxidase system as no superoxide release was induced in

220 neutrophils allowed to interact with the FFA2R modulator (Fig 2). Also the natural FFA2R

221 agonist acetate was shown to be a poor activator of the neutrophil NADPH-oxidase (Fig 2; see

222 also (23)), as compared to the responses induced by the FPR agonists fMLF (FPR1 agonist)

223 and WKYMVM (FPR2 agonist; Fig 2). However, when combined with Cmp58, the NADPH-

224 oxidase activity induced by acetate was largely augmented (Fig 3). The increased

225 production/release of superoxide was evident when Cmp58 was added concomitantly with

226 acetate (Fig 3A), but even more so when neutrophils were pre-treated for five min with

227 Cmp58 before stimulation with acetate (Fig 3C). The amplifying effect with the two FFA2R

228 ligands working together was evident even when the order of addition was reversed (first

229 acetate and then Cmp58) but the amount of superoxide released was lower than when the

230 order was first Cmp58 and then acetate (Fig 3B). In addition, a primed response to acetate

231 was achieved also when 4-CMTB replaced Cmp58 as the allosteric amplifier, but the priming

232 effect was substantially lower than that induced by Cmp58 (Fig 3C inset).

233 The increase of neutrophil superoxide release was most pronounced with Cmp58 and when the

234 cells were pre-incubated with the modulator prior to the addition of acetate, and in order to

235 characterize the response, we used the most potent modulator (Cmp58) and partly "clamped"

236 the system by keeping the concentration of the modulator constant (1μM) and the priming

237 (incubation) time fixed to five min. Using this setup, it is clear that superoxide release from

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238   neutrophils activated with acetate was dependent on the concentration of the orthosteric FFA2R

239   agonist with an EC_{50} value of $\approx 100 \mu$M and a full response at around 500μM (Fig 4A). When

240   using an acetate concentration giving a maximum response (1mM) neutrophil superoxide

241   release was dependent on the concentration of Cmp58 with an EC_{50} value of ≈ 250nM, reaching

242   a full response with a concentration of around 1μM (Fig 4B). Regarding the priming time

243   needed to obtain a good modulating effect, time titration experiments showed that a less than

244   two minutes pre-incubation with Cmp58 was sufficient to achieve a full neutrophil response

245   upon addition of acetate (Fig 4C).

246   In quantitative terms the neutrophil superoxide production induced by 1mM acetate in

247   neutrophils pretreated for five minutes with 1μM Cmp58 was of the same magnitude as that

248   induced by the commonly used FPR agonists fMLF and WKYMVM (100nM; Fig 5A). It was

249   also clear that the response induced by the fairly high concentration of FPR agonists were not

250   affected by pre-incubation with Cmp58 (Fig 5B).

251   In order to determine if the presence of high concentration of acetate was needed to activate the

252   oxidase we performed a "wash out" experiment. In brief, neutrophils (10^7/ml) were pre-

253   incubated with acetate (1mM) and the cell/acetate mixture was then diluted in pre-warmed

254   measuring vials containing Cmp58 (1μM), without or combined with acetate (1mM), giving a

255   final concentration of acetate in the cell-samples of 1mM and 0.01 mM, respectively. The

256   NADPH-oxidase activity was determined directly and, whereas the NADPH-oxidase was

257   activated in neutrophils exposed to 1mM acetate during the whole procedure, no response was

258   induced when the concentration of acetate was rapidly reduced to 0.01mM; Fig 5C). Our data

259   thus show that the effect of acetate is rapidly (or rather immediately) lost upon dilution,

260   suggesting that the dissociation of bound agonist (off-rate) is very rapid.

261   **The primed acetate response was inhibited by the FFA2R antagonist CATPB and the**

262   **allosteric modulator primes also the neutrophil response induced by other FFA2R**

263   **agonists**

264   Next we used the FFA2R antagonist CATPB (20), earlier shown to inhibit FFA2R in

265   neutrophils (23), to further determine the receptor involved in the oxidase activity induced by

266   acetate in cells primed with Cmp58. CATPB potently inhibited the response, shown by the fact

267   that the neutrophil activity induced by 1mM acetate was fully inhibited by 0.1μM CATPB (Fig

268   5D). The inhibitory effects were the same when CATPB was added prior to Cmp58 and also

269   three minutes after Cmp58 (data not shown).

270   Small synthetic molecule ligands with improved potency and selectivity for FFA2R have

271   recently become available and we have previously shown that one such specific receptor agonist

272   (Cmp1) triggers an assembly of the NADPH-oxidase and release of superoxide anions in

273   neutrophils (23). Using Cmp58, we now show that apart from the natural agonist acetate, the

274   neutrophil response induced by Cmp1 was also enhanced by Cmp58 (Fig 5E), and this feature

275   was true also for two other natural FFA2R agonists propionate and butyrate were used (Fig 5F).

276   Further, the same pattern was observed when Cmp58 was replaced by 4-CMTB as shown for

277   Cmp1 induced superoxide release, but the modulating effect of 4-CMTB was lower than that

278   of Cmp58 (Inset Fig 5E). Very low levels of neutrophil NADPH-oxidase activity were induced

279   by propionate/butyrate alone (investigated with concentrations up to 5mM) and the Cmp58

280   primed responses induced by the longer fatty acids were lower than that induced by acetate (Fig

281   5F). The receptor selectivity of the allosteric effect of Cmp58 was investigated and no

282   neutrophil NADPH-oxidase activity was induced in the absence or presence of Cmp58 by the

283   FFA3R selective agonist AR420626 (determined with concentrations up to 5μM; data not

284   shown). The receptor selectivity was, however, not absolute as indicated by the results obtained

285   with low concentrations of the FPR1 agonist fMLF. A neutrophil NADPH-oxidase activity was

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286   induced in the presence of Cmp58 when neutrophils were activated by a concentration of the

287   FPR1 that was non-activating in the absence of the modulator. The Cmp58 mediated

288   augmentation of the fMLF response was not as pronounced as that of the acetate response (Fig

289   5G).

290   Our data show that the pre-incubation time with Cmp58 required to obtain a full neutrophil

291   response upon addition of acetate is very short (Fig 5C). In order to determine if the effect of

292   Cmp58 is reversible, neutrophils (10^7/ml) were pre-incubated with Cmp58 and the

293   cell/compound mixture was then diluted in pre-warmed measuring vials with or without Cmp58

294   (0.5 μM), i.e., the final concentration of Cmp58 in these cell-samples were 0.5 and 0.005 μM

295   Cmp58, respectively, when neutrophils were challenged with acetate. Whereas neutrophils

296   exposed to 0.5μM Cmp58 during the whole procedure were fully responsive to acetate, no

297   neutrophil response was induced by acetate when the concentration of the allosteric modulator

298   was rapidly reduced (from 0.5 to 0.005μM) through the dilution procedure (Fig 5C inset), and

299   the priming effect disappeared already after 30 seconds. Our data thus show that the Cmp58

300   effect is reversible and, more importantly, that one of the mechanisms suggested to be involved

301   in neutrophil priming could be excluded. In many studies a direct link has been described,

302   between priming and recruitment to the cell surface, of receptors stored in easily mobilized

303   secretory organelles (38, 39), and such a mobilization is irreversible (40).

No inhibition of down-stream signaling of FFA2R is induced/mediated by the selective

Gαq inhibitor YM-254890

306   It has been suggested/shown that FFA2R can couple to both Gαi and Gαq for down-stream

307   signaling (17, 41, 42) suggesting that a possible mechanism for the priming effect of the

308   allosteric modulation of Cmp58 on the FFA2R response could be at the level of the signaling

309   G-protein. To elucidate this possibility, we applied the selective Gαq inhibitor YM-254890

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310   which inhibits the down-stream signaling of the neutrophil PAFR whereas no effect is obtained

311   on the response induced by agonist occupied FPRs (Fig 6A and (43)). The Gαq inhibitor YM-

312   254890 had no major effect on Cmp1/FFA2R mediated NADPH-oxidase activity, and the

313   inhibitory profile was the same in Cmp58 primed neutrophils (Fig 6B and C). It has been well

314   established that neutrophil chemotactic GPCRs constitute a receptor class that is coupled to a

315   pertussis toxin-sensitive G protein (44), and we show that also the FFA2R response was

316   sensitive to pertussis toxin treatment. The cells treated with pertussis toxin were thus non-

317   responding to acetate/Cmp1 combined with Cmp58 but fully responsive to PMA (a ROS

318   inducer that signals independent of a G-protein; Fig 6D).

319   The fairly limited effect of the Gαq inhibitor was seen also when looking at the rise in $[Ca^{2+}]_i$

320   induced by acetate in the absence and presence of Cmp58 (Fig 7) evidently demonstrating that

321   Gαq does not play a role in the activity induced in neutrophils by FFA2R agonists.

TNF-α further primed the Cmp58/acetate-triggered activation of the neutrophil NADPH-oxidase

324   Neutrophils treated with the pro-inflammatory cytokine TNF-α exposed an increased number

325   of receptors such as complement receptor 3 (CR3; CD11b) i.e., TNF-α induces mobilization of

326   receptor storing organelles and an increased exposure of receptors to the cell surface (Fig 8A

327   and (23). No NADPH-oxidase assembly and activation was obtained when neutrophils were

328   challenged with TNF-α and this is in agreement with our earlier reported results (23). In

329   addition, no oxidase activity was induced in TNF-α treated cells by Cmp58 or acetate when

330   added alone in concentrations up to 1μM and 1 mM, respectively (data not shown). TNF-α was,

331   however, an additional priming agent when the two FFA2R ligands were combined (Fig 8B).

332   Previous studies have shown that many neutrophil chemoattractant receptors are stored in

333   secretory organelles of naïve cells (38, 45, 46), suggesting that mobilization of these receptors

334   could be a contributing mechanism in priming. However, no increased surface exposure of

335   FFA2R was induced by TNF-α (Fig 8C) indicating that FFA2R is not stored in TNF-α

336   mobilized neutrophil granules.

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Discussion

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343 The allosteric FFA2R modulator Cmp58 ((S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-

344 phenylthiazol-2-yl)butanamide) primes neutrophils in their response to acetate as well as the

345 other natural FFA2R agonists propionate and butyrate. The molecular mechanisms underlying

346 neutrophil priming has been extensively studied and a large number of different mechanisms

347 have been suggested (38, 47-52). By showing that Cmp58 changes the natural FFA2R agonist

348 acetate from being non-activating to a potent neutrophil activating agonist that triggers an

349 assembly of the superoxide generating NADPH oxidase and lowers the thresh-hold for the

350 FFA2R-induced intracellular Ca^{2+} release, we add allosteric receptor modulation as a novel

351 receptor specific neutrophil priming mechanism. The priming agent Cmp58 (22) has earlier

352 been shown to be an FFA2R modulating agonist that binds to an allosteric site on the receptor,

353 and it has also been demonstrated that binding of Cmp58 co-operatively modulate the activity

354 of natural receptor specific ligands in cells other than neutrophils (22). This type of co-

355 operativity between the allosteric agonist Cmp58 and the orthosteric agonist acetate fits with

356 the neutrophil priming data described in this study, except that no direct neutrophil activation

357 was obtained with the allosteric compound in the concentration range tested. We have earlier

358 shown that FFA2R agonists cross desensitizes each other (23); the Cmp58 induced priming is

359 thus not due to an additive effect of two FFA2R agonists. The suggested mechanism for how

360 allosteric modulators affect receptor function involves a direct interaction of the modulator with

361 a specific allosteric binding site and this interaction secondarily transfers the receptor either i)

362 to a state possessing a lower energy barrier for the conformational change required for a shift

363 from a resting non-signaling receptor state to an activated signaling state and by that an

364 increased signaling activity downstream of the agonist occupied receptor, ii) to a state in which

365 the orthosteric binding site has a higher affinity in binding to conventional agonists or, iii)

366 through a combination of the first two (53). It is clear from the dilution experiment performed

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367 (see Fig 5C) that the dissociation of acetate from its receptor is very rapid, suggesting that it is

368 very hard to perform conventional binding experiments with this receptor agonist (54). It has,

369 however, been shown, using a three-way radio-ligand binding experiment, that the ability of

370 acetate to inhibit receptor-binding of an FFA2R antagonist ([^{3}H]GLPG0974) is increased by

371 allosteric FFA2R modulators/agonists (41). Based on these findings it was suggested that an

372 increased binding affinity for orthosteric FFA2R agonists is part of the allosteric effect (41),

373 but irrespectively of the precise modulation mechanism for Cmp58, the outcome of the

374 modulating effect in neutrophils is that acetate is turned into an agonist that activates neutrophils

375 to generate and release superoxide anions.

376 There has been an urgent need for receptor specific tools to allow studies of FFA2R in

377 neutrophils, and among the ligands identified (29), the agonist/antagonist pair Cmp1 and

378 CATPB, fulfilling the requirements of being both potent and receptor selective, were recently

379 used by us to disclose the basic functional characteristics of FFA2R in phagocytic cells (23).

380 Briefly, we showed that Cmp1 triggered an increase in the cytosolic concentration of Ca^{2+} and

381 following this, neutrophils were desensitized not only to Cmp1 but also to the naturally

382 occurring FFA2R agonist acetate, and the antagonist CATPB selectively inhibited the

383 responses. Moreover, the functional and regulatory differences between FFA2Rs and another

384 group of pattern recognition receptors (i.e., the FPRs) imply different roles of these receptors

385 in the orchestration of inflammation and confirm the usefulness of selective FFA2R agonists

386 and antagonists as tools for the exploration of the precise functions of FFA2R. Activation of

387 neutrophils with the FFA2R agonist Cmp1 induced an assembly of the superoxide generating

388 NADPH-oxidase but no chemotaxis, and despite an induction of a rise in intracellular Ca^{2+} by

389 Cmp1, this response was not accompanied by any mobilization of secretory vesicle proteins or

390 cleavage of L-selectin from the cell surface, the two most sensitive markers for neutrophil

391 activation. We now introduce an allosteric modulator as a new receptor specific tool that can

392   be used to study FFA2R in neutrophils. A new concept in the regulation of GPCR signaling

393   was introduced when allosteric ligands were identified and shown to affect (positive or

394   negative) receptor functions when triggered by conventional ligands that interact with the

395   orthosteric binding site exposed by the targeted receptor (53, 55). This concept also adds

396   complexity to the pharmacology and signal transduction scheme downstream an activated

397   receptor, and many different models of how to categorize and analyze this type of allosterism

398   has been described (28, 30). It is clear from the data presented that the FFA2R selective positive

399   allosteric modulator used by us (Cmp58) as well as other modulators such as 4-CMTB (56) and

400   AZ1729 (12) affects the response induced by FFA2R specific orthosteric agonists. The 4-

401   CMTB and AZ1729 compounds have been shown to be both agonists and modulators when

402   interacting with mouse and human neutrophils, respectively (41, 56). The lack of direct

403   neutrophil activating effects of Cmp58/4-CMTB in our systems might be due to species

404   differences, shown to be of importance both for ligand recognition and for signaling of other

405   GPCRs (6, 57) or to differences between the modulating compounds and/or the read-out

406   systems used. Although 4-CMTB is an allosteric modulator it has been shown to have unique

407   properties (24) that could possibly explain why the positive allosteric effect mediated by 4-

408   CMTB on the neutrophil response induced by Cmp1 (Fig 5D) is not evident when determined

409   in other cells or read-out systems (20). The maximal modulating effect of Cmp58 was reached

410   at a concentration of 1μM, suggesting that the allosteric binding sites are fully occupied at this

411   concentration. The lack of direct neutrophil activating effects of Cmp58 when added alone even

412   at a concentration of 5μM, suggests that it should be classified as an allosteric modulator (rather

413   than an allosteric agonist) in relation to FFA2R when the receptor is expressed in neutrophils.

414   The response induced in neutrophils by Cmp58/acetate was abolished in pertussis toxin treated

415   neutrophils, with the logical conclusion that FFA2R signals through a Gαi containing G-

416   protein. We have, however, recently shown that also the neutrophil response mediated by the

417 agonist occupied Gq-coupled receptors for platelet activating factor is inhibited by pertussis

418 toxin, showing that pertussis toxin experiments are not always reliable (43). It has been

419 suggested/shown that FFA2R can couple not only to Gαi but also to Gαq for down-stream

420 signaling (17, 41, 42), but the Cmp58/acetate response was insensitive to the Gq inhibitor YM-

421 254890. Taken together, these data suggest that the signaling cascade triggered by FFA2R and

422 leading to a rise in intracellular Ca^{2+} and activation of the NADPH-oxidase in neutrophils do

423 not involve Gαq, but rather is initiated by a Gαi containing G-protein.

424 Irrespectively of the precise molecular mechanism for the Cmp58 mediated modulation of

425 FFA2R, the functional outcome is that the neutrophil response induced by conventional

426 receptor agonists is primed, and this was evident also in TNF-α treated cells. It is clear from

427 earlier studies that a prominent function of TNF-α is the capacity to in itself prime neutrophils

428 in their response to other stimuli, and we have earlier suggested that mobilization of receptor

429 storage organelles that is induced by the cytokine is an important mechanism in neutrophil

430 priming. Our data showing a lack of FFA2R mobilization in TNF-α primed neutrophils do,

431 however, not support this suggestion. It is clear that the receptor storing organelles mobilized

432 in TNF-α primed neutrophils contain also the flavocytochrome b_{558} (58), the membrane

433 component of the superoxide generating NADPH-oxidase, and mobilization of this protein may

434 well be part of the TNF-α priming process (59). Since augmentation mediated by Cmp58 is

435 reversible, whereas mobilization of neutrophil secretory organelles achieved through a fusion

436 of granule/vesicle membranes with the plasma membrane is a non-reversible process that stably

437 mobilizes proteins from granule/vesicle membranes to the plasma membrane (40), receptor

438 mobilization is not a process directly involved in the Cmp58 induced priming. Regardless of

439 the precise mechanisms for TNF-α priming it is clear that the allosteric modulator adds to the

440 TNF-α effect in making acetate an even more potent NADPH-oxidase activating agonist. The

441 type of allosteric modulation presented in this study, is a novel avenue for potential regulating

442   effects of G-protein coupled receptors expressed in neutrophils. They may offer greater receptor

443   selectivity by binding to non-conserved regions of the receptor among a family of related

444   receptors that may offer other potential advantages such as differential effects on tachyphylaxis

445   and efficacy. Positive allosteric modulators/agonists thus offer an attractive therapeutic

446   approach for the regulation of GPCRs (30). It is clear from the data presented that the neutrophil

447   response is affected by Cmp58 when triggered by sub-optimal concentration of an FPR1

448   agonist. There are several possible mechanisms to this effect, including a receptor dimerization

449   that has been described to modulate fatty acid sensing by FFA2R/FFA3R heterodimers when

450   expressed in monocytes/macrophages (60). The fact that no neutrophil response is induced by

451   the FFA3R agonist AR420626 support an earlier finding, namely that this receptor is not

452   expressed in neutrophils (17), but this does not exclude receptor cross talk between FFA2R and

453   other receptors expressed in these cells. The precise mechanism by which Cmp58 affects the

454   neutrophil response to non-activating concentrations of the FPR1 agonist, thus, needs to be

455   studied in more detail.

456   We show that Cmp58, an allosteric modulator for FFA2R, triggers signals generated in

457   neutrophils by the combined effect of the modulator and the natural agonist acetate determined

458   as release of superoxide anions. This response is even further increased in TNF-α primed

459   neutrophils. The fact that short chain fatty acids are produced by gut microbes during anaerobic

460   metabolism, has suggested that receptors for such molecules expressed on innate immune cells

461   are of prime importance for regulating inflammatory reactions in the gut (12). However, the

462   fact that tissue-recruited neutrophils are non-responding to the FFA2R agonist (23), suggests a

463   role for the receptor also in other organs implying that the role of FFA2Rs is complex and needs

464   further investigation. The phenylacetamides discovered as a novel class of FFA2R allosteric

465   modulators, positively co-operate with natural FFA2R agonists and prime neutrophils in their

466   response to these agonists, may serve as good tools for further unraveling the physiological

467   functions of the FFA2R and its involvement in various diseases. In addition, and even though

468   the majority of the allosteric modulators described so far have been identified through high-

469   throughput screening processes using different libraries of small molecules, the fact that the

470   natural ligands for FFA2R are free fatty acids generated by the fermenting microbiota in the

471   gut, unavoidably raises the question whether endogenous or other naturally occurring microbial

472   metabolites can fulfill an allosteric function and physiologically change the functional

473   repertoire of FFA2R and other GPCRs.

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Authorship contributions

JM and AH designed and performed research and analyzed data. MS, MG and LB performed research and analyzed data. HF and CD planned and supervised the research, analyzed data, and wrote the manuscript that was edited by all authors.

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669   **Figure legends**

670

671   **Figure 1. The allosteric FFA2R agonist Cmp58 modulates the transient rise in**

672   **intracellular Ca^{2+} induced by acetate in neutrophils**

673   Human neutrophils loaded with Fura-2 were stimulated with acetate (upper panel; different

674   concentrations) or Cmp58 and acetate added in sequence (lower panel; first Cmp58 1μM, and

675   thereafter different concentrations of acetate). No neutrophil response was induced by Cmp58

676   alone (middle panel) and for comparison, the neutrophil response induced by the FPR1 agonist

677   (fMLF; 5nM) is also shown. The time point for addition of an agonist is indicated by an arrow.

678   Abscissa, time of study (sec); Ordinate, Increase in intracellular $[Ca^{2+}]_i$ given by the ratio

679   between Fura-2 fluorescence at 340 and 380 nm.

680

681   **Figure 2. The allosteric FFA2R ligand Cmp58 has no direct effect on the neutrophil**

682   **NADPH-oxidase activity**

683   Human neutrophils were activated with Cmp58 alone (5 μM, dotted line) and the release of

684   superoxide anions was recorded. One representative experiment out of >5 is shown, and the

685   responses induced by the FPR agonists fMFL (100 nM; FPR1 agonist, solid line) and

686   WKYMVM (100 nM; FPR2 agonist, dashed line) are included for comparison. Superoxide

687   production was recorded continuously and the time point for addition of the agonists is indicated

688   by an arrow. Abscissa, Time of study (min); Ordinate, Superoxide production (arbitrary units;

689   Mcpm). **Inset:** The neutrophil response induced by different concentrations of Cmp58 and

690   acetate alone (5mM) are shown and the time point for addition of Cmp58/acetate is indicated

691   by an arrow. Abscissa, Time of study (min); Ordinate, Superoxide production (arbitrary units;

692   Mcpm)

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Figure 3. Neutrophil activation of the NADPH-oxidase by Cmp58 and acetate when added together or in sequence

Human neutrophils were activated with Cmp58 (1μM) and acetate (1mM) and the two FFA2R ligands were added simultaneously (**A**) or in sequence (**B** and **C**). The time points for addition of the molecules are indicated by arrows and superoxide production was recorded continuously. Abscissa, Time of study (min); Ordinate, Superoxide production (arbitrary units; Mcpm). **Inset in C:** The bar graph shows a comparison of the response induced by acetate (1mM) in neutrophils treated for 5 min with Cmp58 (1μM) or 4-CMTB (1μM). The peak responses were determined and the results are given as the ratio between the activities in the presence of 4-CMTB and Cmp58 (mean±SD; n=3).

Figure 4. Dose and time titration of NADPH-oxidase activity induced in neutrophils stimulated with acetate after preincubation with Cmp58

A. Human neutrophils were incubated without and with Cmp58 (1μM) for 5 min at 37°C followed by an activation with acetate at different concentrations. Superoxide production was recorded continuously and the peak values of the responses were determined and compared. Abscissa, Concentration of acetate (mM); Ordinate, Superoxide production expressed in percent of the activity induced by 1mM acetate (mean±SD; n=3).

B. Human neutrophils were incubated without and with different concentrations of Cmp58 as indicated for 5 min at 37°C and then activated by and acetate (1mM). Superoxide production was recorded continuously and the peak values of the responses were determined and compared. Abscissa, Concentration of Cmp58 (μM); Ordinate, Superoxide production given in percent of the activity induced by acetate with neutrophils preincubated with 1μM Cmp58 (mean±SD; n=3).

720 **C.** Human neutrophils were incubated with Cmp58 (1μM) for up to 5 min at 37°C and the cells

721 were then stimulated with acetate (1mM). Superoxide production was recorded continuously

722 and the peak values of the responses were determined and compared to the value obtained with

723 neutrophils pre-incubated for 5 min with Cmp58. Abscissa, pre-incubation time (min);

724 Ordinate, Superoxide production given as ratios with the response induced in neutrophils

725 preincubated with Cmp58 for 5 min (mean±SD; n=3).

726 **Figure 5. Basic characteristics of the augmented NADPH-oxidase activity mediated by**

727 **Cmp58**

728 **A.** Human neutrophils were incubated with Cmp58 (1μM) for 5 min at 37°C and then activated

729 by acetate (1mM; dotted line). Superoxide production was recorded continuously and the

730 response was compared to the activities induced by fMLF (100nM; FPR1 agonist; broken line)

731 and WKYMVM (100nM; FPR2 agonist; solid line). The time point for addition of the agonists

732 is indicated by an arrow. Abscissa, Time of study (min); Ordinate, Superoxide production (in

733 arbitrary units; Mcpm). **Inset**: Human neutrophils were incubated with Cmp58 (1μM) for 5 min

734 at 37°C and then activated by acetate (1mM). Superoxide production was recorded continuously

735 and the response was compared to the activities induced by fMLF (100nM; FPR1 agonist) and

736 WKYMVM (100nM; FPR2 agonist). The peak values of the responses were determined,

737 compared, and are presented as the ratios WKYMVM/Cmp58+acetate (black) and

738 fMLF/Cmp58+acetate (grey), respectively (mean±SD; n=3).

739 **B**. Human neutrophils were incubated without (dotted line) or with (solid line) Cmp58 (1μM)

740 for 5 min at 37°C and then activated with WKYMVM (100nM). Superoxide production was

741 recorded continuously and the time point for addition of the agonists is indicated by an arrow.

742 Abscissa, Time of study (min); Ordinate, Superoxide production (in arbitrary units; Mcpm).

743 **Inset:** Human neutrophils were incubated without or with Cmp58 (1μM) for 5 min at 37°C and

744 then activated by and WKYMVM (100nM or 50nM) or fMLF (100nM or 50nM). Superoxide

34

745 production was recorded continuously, the peak activities were determined and ratios

746 with/without Cmp58 were calculated for 50 and 100nM of the FPR agonists, respectively (ratios

747 fMLF+/-Cmp58, black and WKYMVM+/-Cmp58, grey; mean±SD, n=3).

748 **C**. Human neutrophils were incubated with acetate (1mM) for 5 min and the cells were then

749 diluted 1/200 in measurement vials containing Cmp58 (1μM) without (dashed line) or with

750 (solid line) acetate (1mM) and production of superoxide was recorded directly. One

751 representative experiment out of 3 is shown. Abscissa, Time of study (min); Ordinate,

752 Superoxide production (arbitrary units; (Mcpm). **Inset:** Human neutrophils were incubated with

753 Cmp58 (0.5μM) for 10 min at 37ºC and the cells were then diluted 1/100 in measurement vial

754 without (dashed line) or with Cmp58 (0.5μM, solid line) and the cells (in 0.5 and 0.005μM

755 Cmp58) were activated by acetate 30 seconds after the samples were diluted in new buffer.

756 Superoxide production was recorded continuously and the time point for addition of acetate is

757 indicated by an arrow and one representative experiment out of more than 3 is shown. Abscissa,

758 Time of study (min); Ordinate, Superoxide production (arbitrary units; (Mcpm).

759 **D.** Human neutrophils were incubated with the FFA2R antagonist CATPB at different

760 concentrations as indicated and the activated by the combination of Cmp58 (1μM; 5 min

761 together with CATPB) and acetate (1mM). The neutrophil response was measured as

762 superoxide production that was recorded continuously. The peak activities were determined and

763 the results expressed as percent of the activity induced by Cmp58/acetate in the absence of the

764 FFA2R antagonist. Abscisssa, Concentration of CATPB (log M); Ordinate, remaining activity

765 (in percent of the activity induced without any antagonist; mean±SD, n=3).

766 **E.** Human neutrophils were incubated without (dark grey bars) or with Cmp 58 (1μM; light

767 grey bars) for 5 minutes and then activated with the FFA2R agonist Cmp1 at different

768 concentrations (μM) as indicated. Superoxide production was recorded continuously and the

769 peak activities were determined. Abscissa, Concentration of Cmp1 (μM); Ordinate, Superoxide

770   production (peak value in arbitrary units; (Mcpm; mean±SD, n=3). **Inset**: Human neutrophils

771   were incubated without or with Cmp 58 (1μM) and 4-CMTB (5μM), respectively, for 5 minutes

772   and then activated with the FFA2R agonist Cmp1 (1μM). Superoxide production was recorded

773   continuously and the peak activities were determined and the results are given as fold increase

774   in the presence of the modulators compared to the activity induced by Cmp1 alone. (mean±SD,

775   n=3).

776   **F.** Human neutrophils were incubated with Cmp58 (1μM) for 5 min at 37°C and then activated

777   by acetate, propionate or butyrate (1mM). Superoxide production was recorded continuously

778   and the responses were compared. The responses induced by propionate and butyrate in the

779   absence of Cmp58 are also shown. The peak values of the responses were determined, and

780   given as the ratios acetate/propionate and acetate/butyrate, respectively (mean±SD; n=3).

781   **G.** Human neutrophils were incubated with or without Cmp58 (1μM) for 5 min at 37°C and

782   then activated by and acetate (1mM) or fMLF (1nM). The time point for addition of the agonists

783   is indicated by an arrow. Abscissa, Time of study (min); Ordinate, Superoxide production (in

784   arbitrary units; Mcpm). **Inset**: Human neutrophils were incubated with Cmp58 (1μM) for 5 min

785   at 37°C and then activated by acetate (1mM) or fMLF (1nM). Superoxide production was

786   recorded continuously and the responses were compared. The peak values of the responses were

787   determined, and is shown as the ratio Cmp58+acetate/Cmp58+fMLF (mean±SEM; n=6).

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789   **Figure 6. The FFA2R induced activation of the neutrophil NADPH-oxidase does not**

790   **engage Gαq signaling but is inhibited by pertussis toxin**

791   **A.** To show the receptor selectivity of the Gαq inhibitor YM-254890, human neutrophils were

792   incubated for 5 min (at 37°C) with (dotted lines) or without (solid lines) the the Gαq inhibitor

793   YM-254890 (200 nM), the cells were then activated by the addition of PAF (100 nM) or fMLF

794   (100 nM; inset) and the release of O$_2^-$ was measured over time. Time point for addition of

36   36

795   agonist shown by the arrows. One representative experiment is shown (see also (43)). Abscissa,

796   time of study (min); ordinate, O_2^- production given in arbitrary units (Mcpm).

797   **B.** Human neutrophils were incubated for 5 min at 37^0C with or without the Gαq inhibitor YM-

798   254890 (200 nM) and the cells were then activated by the addition of Cmp1(1 μM) at the time

799   point shown by the arrow and the release of O_2^- was measured over time. One representative

800   experiment out of > 3 is shown. Abscissa, time of study (min); ordinate, O_2^- production given

801   in arbitrary units (Mcpm; 10^6 counts/min). **Inset:** The inhibitory effect of YM-254890 (200nM)

802   on the Cmp1 induced of O_2^- production, presented as percent of the response (peak value) in

803   the absence of the inhibitor (mean ± SD; n = 3).

804   **C.** Human neutrophils were incubated for 5 min (at 37^0C) with Cmp58 together with (dotted

805   line) or as control without (solid line), the Gαq inhibitor YM-254890 (200 nM). The cells were

806   then activated by the addition of the FFA2R agonist Cmp1(1 μM) at the time point shown by

807   the arrow and the release of O_2^- was measured over time. One representative experiment out of

808   three is shown. Abscissa, time of study (min); ordinate, O_2^- production given in arbitrary units

809   (Mcpm). **Inset:** The inhibitory effect of YM-254890 (200nM) given in percent of the control

810   the Cmp1 responses induced in the absence of the inhibitor. The peaks of O_2^- produced were

811   compared (mean ± SD; n = 3).

812   **D**. Human neutrophils were incubated without or with Pertussis toxin (PTX, 500 ng/ml) for 150

813   min at 37°C, the cells were then pre-treated with Cmp58 (1 μM) for 5 minutes before

814   stimulation with Cmp1 (1 μM) or acetate (1 mM). For comparison, PTX treated and untreated

815   neutrophils were activated by fMLF (100 nM) or PMA (50 nM; a non-G-protein dependent

816   stimulus). The peak superoxide activities in the presence and absence of PTX were compared,

817   and the bar graph shows remaining activity following PTX-treatment in percent of the response

818   induced by the agonists in non-treated neutrophils (n = 3; mean±SD).

819 **Figure 7. The acetate induced rise in $[Ca^{2+}]_i$ is not affected by Gαq inhibitor YM-254890**

820 **independent of the presence or absence of the allosteric modulator Cmp58**

821 **A.** Human neutrophils labeled with Fura-2 were used to measure Ca^{2+} transients. The cells were

822 pre-incubated at 37°C for 10 min with (dotted line) or without (solid line) the Gαq inhibitor

823 YM-254890 (200 nM). The neutrophils were then stimulated with acetate (2.5mM; as indicated

824 by the arrow) and the Ca^{2+} transient was measured over time. One representative experiment

825 out of three is shown. Abscissa, time of study; ordinate, increase in intracellular $[Ca^{2+}]_i$ given

826 by the ratio between Fura-2 fluorescence at 340 and 380 nm. **Inset:** The bar graph shows the

827 peak values of acetate induced intracellular $[Ca^{2+}]_i$ in the absence (light grey bar) or presence

828 of the YM-254890 (200 nM dark grey bar; mean±SD; n = 3).

829 **B.** Neutrophils labeled with Fura-2 were used to measure Ca^{2+} transients. The cells were pre-

830 incubated at 37°C for 10 min with Cmp58 (1 μM) combined with (dotted line) or without (solid

831 line) the Gαq inhibitor YM-254890 (200 nM). The neutrophils were then stimulated with

832 acetate (0.25mM, as indicated by the arrow) and the Ca^{2+} transient was measured over time.

833 One representative experiment out of three is shown. Abscissa, time of study; ordinate, increase

834 in intracellular $[Ca^{2+}]_i$ given by the ratio between Fura-2 fluorescence at 340 and 380 nm. **Inset:**

835 The bar graph shows the peak values of cmp58+acetate induced intracellular $[Ca^{2+}]_i$ induced in

836 the absence (light grey bar) or presence of the inhibitor YM-254890 (200nM, dark grey bar;

837 mean ± SD; n = 3).

838 **Figure 8. TNF-α further primes the neutrophil response induced by acetate in the**

839 **presence of the allosteric modulator Cmp58**

840 **A.** The histogram shows the neutrophils cell-surface expression of CR3 after incubation without

841 (light grey) or with TNF-α (10 ng/mL) for 20 min at 37°C, as measured by flow cytometry.

842   **Inset**: The bar graph shows the mean fold increase in fluorescence for primed cells (TNF-

843   α, black bar) as compared to naïve (non-primed) cells (grey bars; mean±SD; n=3).

844   **B.** Neutrophils pre-incubated for 15 min at 37°C without (dotted line) or with (solid line) TNF-

845   α (10 ng/ml) were then incubated for another 5 minutes with Cmp58 (1μM) and activated with

846   the FFA2R agonist acetate (1mM). Superoxide production was recorded continuously and one

847   representative experiment out of three is shown. Abscissa, Time of study (min); Ordinate,

848   Superoxide production (peak value in arbitrary units; Mcpm). **Inset:** The peak NADPH-oxidase

849   values were determined and the bar graph shows the ratio between the value obtained with

850   TNF-α primed cells compared to that obtained with naïve (non-TNF-α–treated) cells

851   (mean±SD; n=3).

852   **C.** The histogram shows the neutrophils cell-surface expression of FFA2R after incubation

853   without (light grey) or with TNF-α (10 ng/mL) for 20 min at 37°C, as measured by flow

854   cytometry. **Inset**: The bar graph shows the fold of FFA2R expression for primed cells (TNF-

855   α, black bar) as compared to non-primed cells (grey bars; mean±SD; (n=3).

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Fig. 1 Mårtensson et al.

Neutrophils stimulated with acetate alone (mM)

0.05   0.10   0.25   0.63   1.25   2.50

Neutophils stimulated with Cmp 58 alone (μM), fMLF as control

1.00   5.00   fMLF

Cmp 58 pre-treated neutrophils stimulated by acetate (mM)

0.05   0.10   0.25   0.63   1.25   2.50
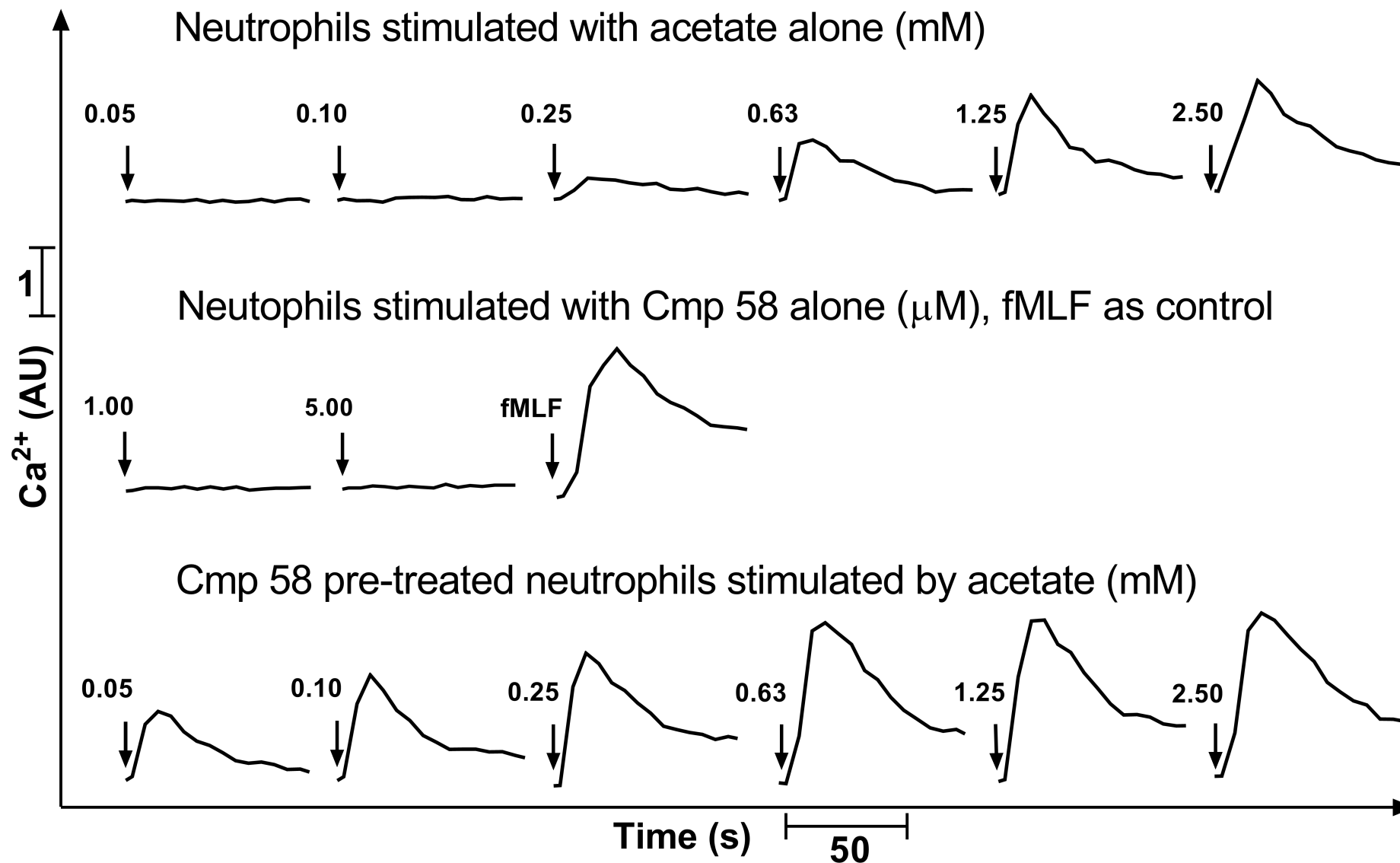
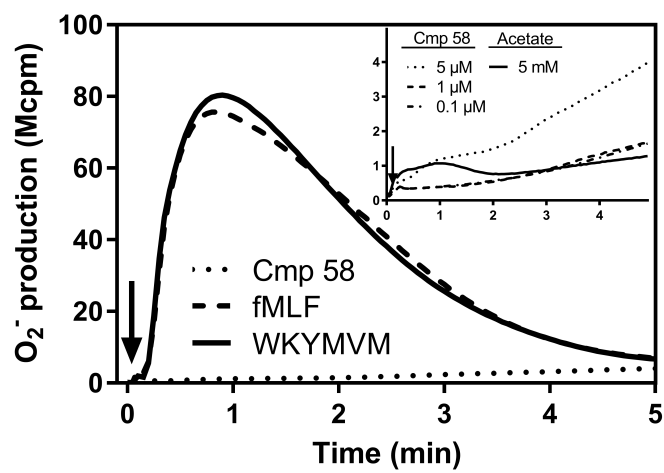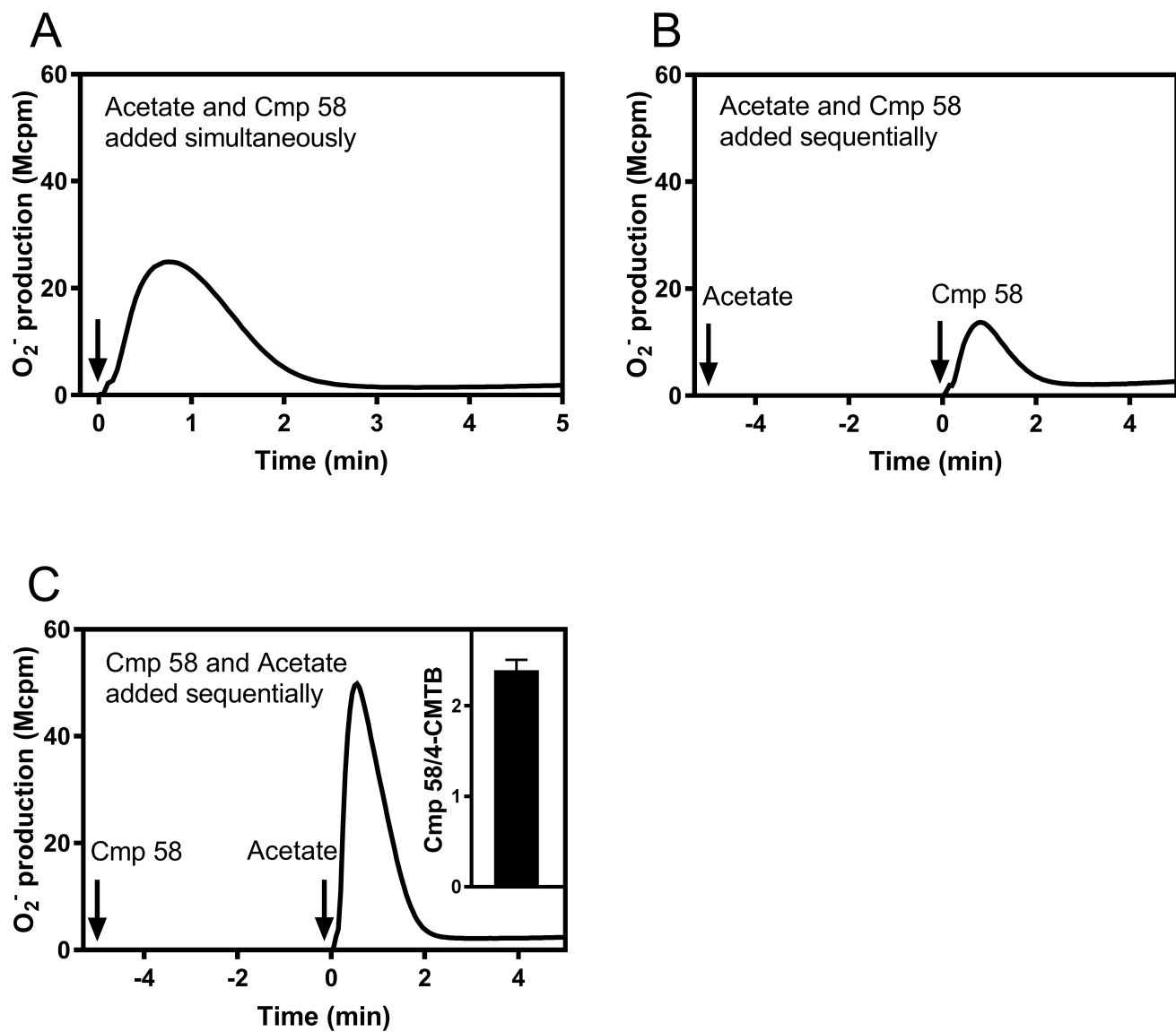Ca²⁺ (AU)

1

Time (s)   50

Fig. 2 Mårtensson et al.

Fig. 3 Mårtensson et al.

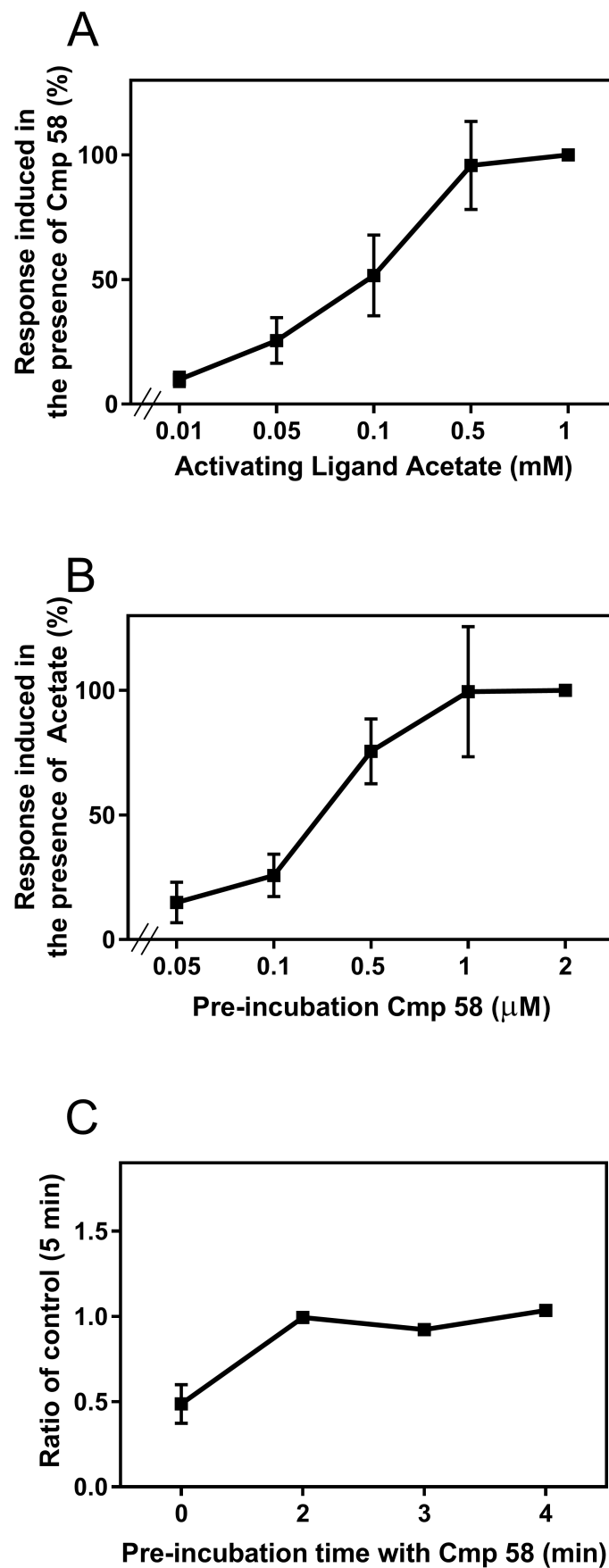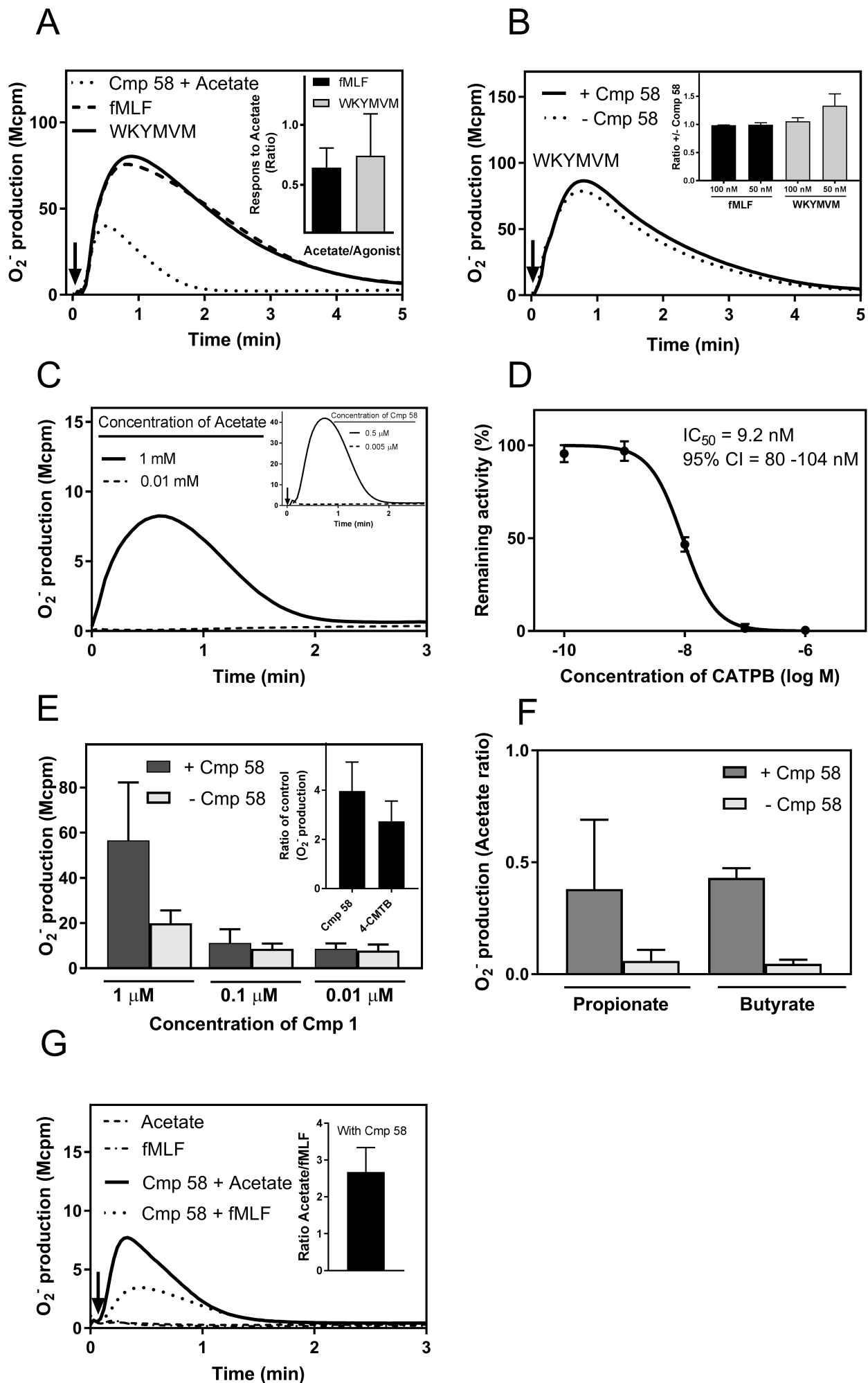# Fig. 4 Mårtensson el al.
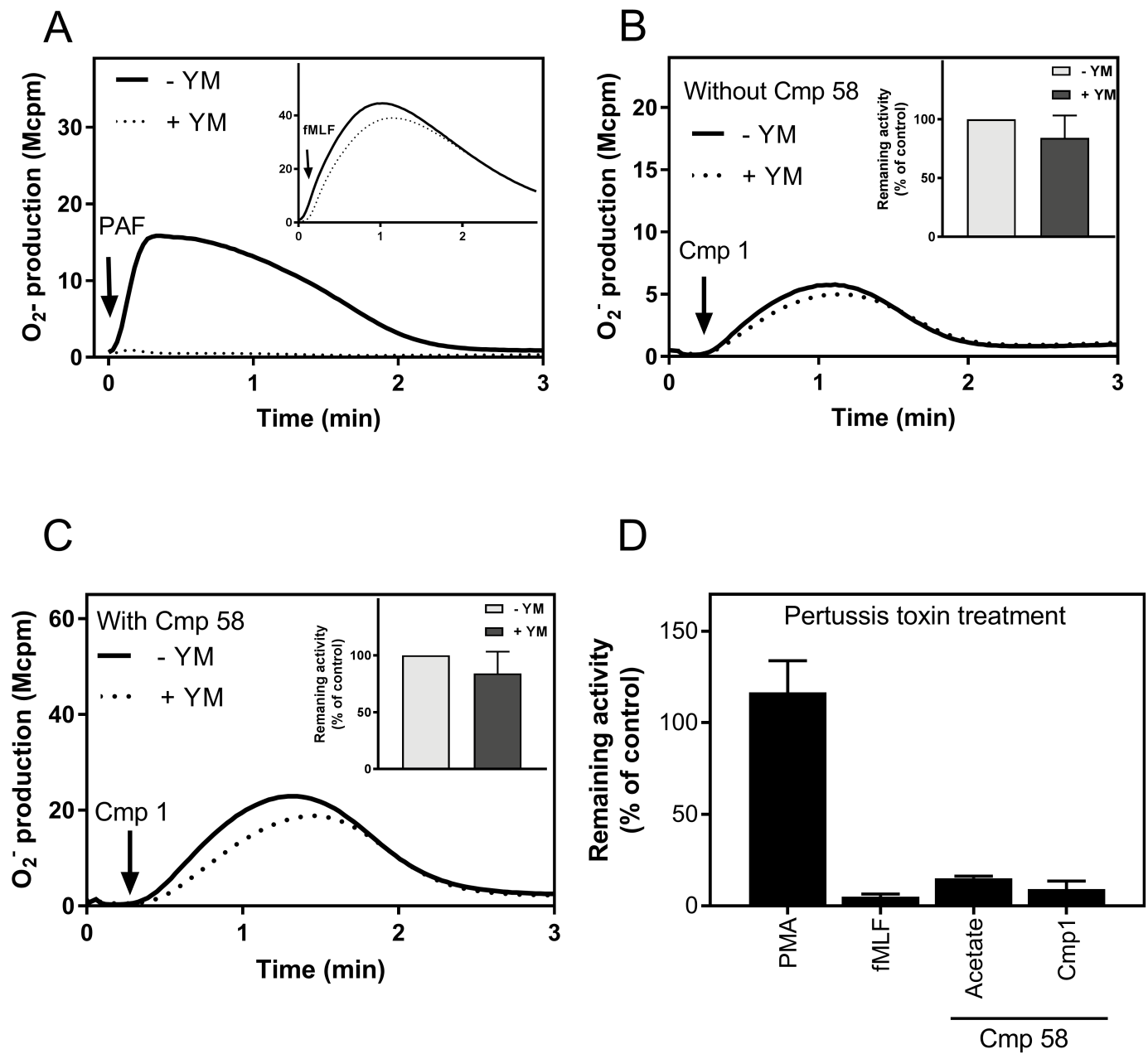
Fig. 5 Mårtensson et al.

Fig. 6 Mårtensson et al.

Fig. 7 Mårtensson et al.

Fig. 8 Mårtensson et al.