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IL-1 β and TNF α inhibit GPR120 (*FFAR4*) and stimulate GPR84 (*EX33*) and GPR41 (*FFAR3*) fatty acid receptor expression in human adipocytes: implications for the anti-inflammatory action of *n*-3 fatty acids

Laura Muredda¹, Malgorzata A. Kępczyńska¹, Mohamed S. Zaibi^{1a}, Suliman Y. Alomar², Paul Trayhurn^{1,2,3}

^a*Clore Laboratory, University of Buckingham, Buckingham, United Kingdom,* ²*Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia, and* ³*Obesity Biology Unit, University of Liverpool, Liverpool, United Kingdom*

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*Correspondence should be addressed to:

Professor Paul Trayhurn, FRSE

Clore Laboratory

University of Buckingham

Hunter Street

Buckingham MK18 1EG, UK

Email: p.trayhurn@liverpool.ac.uk

Fax: +44 1280 820135

Abstract

Regulation of the expression of GPCR fatty acid receptor genes has been examined in human adipocytes differentiated in culture. TNF α and IL-1 β induced a marked reduction in *GPR120* expression, mRNA level falling 17-fold at 24h in adipocytes incubated with TNF α . In contrast, *GPR84* mRNA was dramatically increased by these cytokines (>500-fold for IL-1 β at 4h); *GPR41* expression was also stimulated. Rosiglitazone did not affect *GPR84* expression, but *GPR120* and *GPR41* expression increased. Dexamethasone, insulin, linoleic and docosahexaenoic acids, and TUG891 (*GPR120* agonist) had little effect on *GPR120* and *GPR84* expression. TUG891 did not attenuate the pro-inflammatory actions of TNF α and IL-1 β . Docosahexaenoic acid slightly countered the actions of IL-1 β on *CCL2*, *IL6* and *ADIPOQ* expression, though not on secretion of these adipokines. *GPR120* and *GP84* gene expression in human adipocytes is highly sensitive to pro-inflammatory mediators; the inflammation-induced inhibition of *GPR120* expression may compromise the anti-inflammatory action of *GPR120* agonists.

Introduction

Several members of the extended family of G-protein-coupled receptors (GPCR) are specific sensors/receptors for fatty acids, each of which is selective for different types of fatty acid. GPR40 (also known as FFAR1) is activated by saturated and unsaturated medium and long chain fatty acids (C8-C22) with GPR41 (FFAR3) and GPR43 (FFAR2) being receptors for short chain fatty acids (C1-C6) (Milligan *et al.*, 2014; Watterson *et al.*, 2014; Ulven & Christiansen, 2015). GPR84 (EX33) is activated primarily by medium chain fatty acids (C9 to C14 are the most potent), while GPR120 (FFAR4) is considered as a receptor for *n*-3 polyunsaturated fatty acids (PUFA), although it is also activated by medium and long chain fatty acids (Oh *et al.*, 2010; Milligan *et al.*, 2014; Watterson *et al.*, 2014). These GPCRs have been linked to a range of metabolic effects, activation of GPR40, for example, leading to the stimulation of glucose-dependent insulin secretion by pancreatic β cells (Itoh *et al.*, 2003), while activation of GPR43 by short chain fatty acids stimulates the release of leptin from adipocytes and of GLP-1 from colonic cells (Zaibi *et al.*, 2010; Tolhurst *et al.*, 2012).

GPR84, which is the least studied of the GPCR fatty acid sensors, is reported to be a pro-inflammatory receptor, activation by medium chain fatty acids resulting in chemotaxis and cytokine production by macrophages (Suzuki *et al.*, 2013). Activation of GPR120 by *n*-3 PUFA, on the other hand, has been linked to the insulin-sensitising and anti-inflammatory actions of these fatty acids, particularly in relation to obesity (Oh *et al.*, 2010; Oh & Olefsky, 2012; Ulven & Christiansen, 2015; Moniri, 2016). GPR120 knockout mice become obese on a high fat diet and exhibit multiple metabolic disorders, including insulin resistance associated with reduced insulin signalling and enhanced inflammation in white adipose tissue (Ichimura *et al.*, 2012). Other functions attributed to GPR120 in adipose tissue include the promotion of adipogenesis (Song *et al.*, 2016) and the stimulation of VEGF-A production by mature adipocytes (Hasan *et al.*, 2015).

Each of the GPCRs for fatty acids exhibit selective tissue and cellular distribution in their expression. The *GPR120* gene is widely expressed, but expression is particularly evident in adipose tissue, both in adipocytes themselves and in M1 and M2 macrophages (Oh *et al.*, 2010). *GPR84* is also expressed in adipocytes, as well as in leukocytes and other immune cells (Wang *et al.*, 2006; Nagasaki *et al.*, 2012). *GPR84* expression in monocytes/macrophages has been shown to be markedly induced by lipopolysaccharide (Wang *et al.*, 2006), while in adipocytes both macrophage secretions and TNF α stimulate expression (Nagasaki *et al.*, 2012; Trayhurn & Denyer, 2012). In contrast, macrophage secretions appear to down-regulate *GPR120* expression (Trayhurn & Denyer, 2012). These observations suggest that GPR84 and GPR120 are intimately connected to the inflammatory response in adipose tissue which is evident in obesity, the obese

state being characterised by chronic inflammation in the tissue (Rajala & Scherer, 2003; Trayhurn & Wood, 2004; Hotamisligil, 2006). Adipose tissue inflammation is widely considered to underlie the development of several obesity-associated disorders, particularly insulin resistance and the metabolic syndrome (Hotamisligil, 2006; Rosen & Spiegelman, 2006; Bluher, 2009).

The aim of the present study was: (i) to examine the regulation of *GPR120* and *GPR84* expression in human adipocytes by a range of factors, both pro-inflammatory and anti-inflammatory, and including GPR120 agonists; (ii) to examine the effect of GPR120 agonists, synthetic and natural, on the inflammatory response induced by TNF α and IL-1 β in human fat cells. The results demonstrate that both TNF α and IL-1 β markedly stimulate the expression of *GPR84*, but inhibit *GPR120* expression, and that agonists to GPR120 have little or no effect on the stimulation of inflammation by these cytokines in human adipocytes.

Materials and methods

Adipocyte cell culture

Human fibroblastic pre-adipocytes were purchased from PromoCell (Germany; Catalogue #C-12730, Lots #400Z008.1 and 404Z027.2), together with proprietary cell culture media. The pre-adipocytes were isolated from the subcutaneous adipose tissue of Caucasian females (aged 29 and 46 years). As previously described, the pre-adipocytes were plated into 12-well plates (5,000 cells/cm²), cultured to confluence, differentiated into adipocytes and then further cultured for up to 14 days (Alomar et al., 2015). In outline, the pre-adipocytes were first cultured in a growth medium containing 5% foetal calf serum, epidermal growth factor (10 ng/ml), hydrocortisone (1 μ g/ml) and heparin (90 μ g/ml). They were then transferred to a differentiation medium (without foetal calf serum) for 72 h, this medium containing IBMX (44 μ g/ml), thyroxine (9 ng/ml), dexamethasone (400 ng/ml), insulin (0.5 μ g/ml) and the PPAR γ agonist rosiglitazone (3 μ g/ml). The differentiating cells were finally incubated in a nutrition medium which contained 3% foetal calf serum, dexamethasone (400 ng/ml) and insulin (0.5 μ g/ml); this growth medium was changed every 2-3 days. The differentiated adipocytes were used at between 12 and 14 days after differentiation was induced, by which time they contained multiple lipid droplets.

To assess the effects on GPCR gene expression, the fat cells were incubated for either 4 or 24 h with one of the following agents: human recombinant IL-1 β (0.5 or 2 ng/ml; Sigma, UK), TNF α (5 and 100 ng/ml; Sigma, UK), rosiglitazone (0.1 and 1 μ M; Sigma, UK), dexamethasone (2 and 20 nM; Sigma, UK), insulin (1 and 20 nM; Sigma, UK) or TUG891 (0.5 and 10 μ M; University of Southern Denmark, Denmark). Control cells received vehicle. In studies investigating the effects of insulin and dexamethasone, these hormones were removed from the

culture medium 24 h before the start of the experiment (and were absent from the control cells). For experiments with the fatty acids linoleic acid and docosahexaenoic acid (DHA), two concentrations were used; 25 and 100 μ M for DHA, and 50 and 200 μ M for linoleic acid. The fatty acids were added to the nutrition medium which was supplemented with 0.3% bovine serum albumin.

At the end of the incubation period, the culture medium was aspirated and stored at -20°C ; the adipocytes were washed and frozen in TRI Reagent (Sigma, UK) and stored at -80°C . A total of 3-6 individual sets of cells was taken for each experimental group.

RNA extraction and real-time PCR

The adipocytes were thawed on ice, homogenised in the TRI Reagent in which they had been stored and total RNA extracted using an RNeasy Micro Kit (Qiagen, UK). The purity of the RNA was close to 2.0, based on the 260/280 nm and 260/230 nm ratios (NanoDrop 1000; Wilmington, USA). The RNA Integrity Number (Agilent 2100 Bioanalyser; Agilent Technologies, Germany) was approximately 10.

The total RNA was DNase-treated with a TURBO-DNA-freeTM kit (Ambion, Life TechnologiesTM, USA) and 0.8-1.6 μ g was reverse transcribed using *Taqman*[®] reverse transcription reagents (InvitrogenTM, Applied Biosystems, UK). Between 60 and 80 ng of cDNA was taken for real-time PCR which was performed in triplicates using Gene Expression Master Mix and *TaqMan*[®] Gene Assays consisting of specific *Taqman*[®] probes (Applied Biosystems[®], Life TechnologiesTM, USA). Probes were obtained for the human *GPR40* (Hs03045166_s1), *GPR41* (Hs02519193_g1), *GPR43* (Hs00271142_s1), *GPR84* (Hs01874713_s1), *GPR120* (Hs00699184_m1), *CCL2* (Hs00234140_m1), *IL1B* (Hs01555410_m1), *IL6* (Hs00985639_m1), *IL16* (Hs00189606_m1) and *ADIPOQ* (Hs00605917_m1) genes; probes were also obtained for *ACTB* (β -actin; Hs99999903_m1) as the control gene. PCR reactions were set up in duplex format where the FAM-labelled *Taqman*[®] probe for the gene of interest was mixed with the VIC-labelled *Taqman*[®] probe for *ACTB*.

PCR amplification was performed using an ABI real-time PCR detection system (ABI StepOneplusTM, Applied Biosystems[®], USA) with a two-step thermal cycling: 95°C for 10 min, followed by 40 cycles of 95°C for 15sec and 60°C for 1 min. The data were analysed by the comparative $2^{-\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen, 2001) and expressed as fold-changes in the target gene (normalized to *ACTB* as the reference gene) in treated adipocytes and related to the expression of the control adipocytes (normalised to the mRNA level in the control cells = 1.0).

PCR arrays

PCR arrays were performed essentially according to the manufacturer's instructions, as described previously (Alomar *et al.*, 2015; Kepczynska *et al.*, 2017). Extracted RNA was DNase-treated, reverse transcribed using a RT² First Strand Kit (Qiagen, UK) and then screened with a RT² Profiler PCR array for 84 Human Cytokine and Chemokine genes (Qiagen; Catalogue #PAHS-150ZC-24). PCR amplification was performed by real-time PCR (ABI StepOneplus) with two-step thermal cycling using the following protocol; 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min. The data were analysed by the comparative 2^{-ΔΔCt} method (Livak & Schmittgen, 2001) and expressed as fold-changes in the target gene normalised to the reference genes (*ACTB*, *B2M*, *GAPDH*, *HPRT*, *RLPO*) for the adipocytes treated with TNFα and related to the expression level in the control cells.

Protein measurements

The concentration of the adipokines IL-6, IL-16, MCP-1 (encoded by *CCL2*) and adiponectin was measured in the medium using MSD immunoassays (Meso Scale Discovery, USA); these assays enable the rapid and sensitive measurement of specific proteins in small sample volumes. Before analysis, the media were centrifuged to remove any cell debris. Plates were pre-coated with antibodies on independent, discrete spots and the assay then performed essentially according to the manufacturer's instructions, as described previously (Alomar *et al.*, 2015; Alomar *et al.*, 2016). The data was analysed using Proprietary Meso Scale software. The lowest level of detection was 0.06 pg/ml for IL-6 (interlot CV <20 %), 2.83 pg/ml for IL-16 (interlot CV <20 %), 0.09 pg/ml for MCP-1 and 5 pg/ml for adiponectin (mean intraplate CV <20 % for MCP-1 and adiponectin).

Statistical analysis

The statistical significance of differences between groups was assessed by one-way ANOVA with Bonferroni post-test (for selected groups) or with Student's unpaired *t* test; a value of *P*<0.05 was taken as being statistically significant.

Results

Regulation of GPR120 and GPR84 gene expression

In the first set of studies, the effect of a series of hormones and other factors on the expression of the *GPR120* and *GPR84* genes was examined in human adipocytes differentiated in culture from fibroblastic pre-adipocytes. The expression of the *GPR41* gene was also explored. Both acute (4 h) and sustained (24 h) responses were examined, and a low and a high concentration of the agent were generally employed, based on previous work (Peeraully *et al.*, 2004; Gao & Bing,

2011; Alomar *et al.*, 2015). The initial studies focused on the effects of the pro-inflammatory cytokines TNF α and IL-1 β .

Incubation with TNF α resulted in a dose-dependent reduction in GPR120 mRNA level at both 4 and 24 h of treatment (Fig 1A). At 4 h, GPR120 mRNA level fell with the higher dose to 22% of the control cells, while at 24 h it was reduced to just 6% of controls – a 17-fold reduction. In marked contrast to GPR120, there was a major stimulation of *GPR84* expression by TNF α (Fig 1A), and as with GPR120 this was dose-dependent at both time-points. The increase in GPR84 mRNA was greater at 4 than at 24 h, the level being ~200-fold greater relative to the controls with the higher dose at 4 h; surprisingly, the 46-fold increase in GPR84 mRNA at 24 h with the high dose was not statistically significant due to the considerable variation observed.

The effect of TNF α on the expression of *GPR40*, *GPR41* and *GPR43* was also determined. There was a marked stimulation of *GPR41* expression at 24 h, the mRNA level being >30-fold higher with both the low and high doses of the cytokine (Fig 1A). There was also an increase at 4 h, albeit much lower, at just 4-fold relative to the control cells (Fig 1A). Modest, but statistically significant, increases in GPR40 mRNA level at 4 h (both doses) and at 24 h for the low, though not the high, dose of TNF α were evident (results not shown). GPR43 mRNA level was not significantly increased by TNF α at 4 h, but there was a significant increase at 24 h and this was greater for the low dose (11-fold) than the high (5-fold) (results not shown).

The effects of IL-1 β were examined using a single high concentration, based on previous studies on human adipocytes with this cytokine (Alomar *et al.*, 2015). IL-1 β had broadly similar effects to TNF α , with a reduction in GPR120 mRNA level and an increase in GPR84 mRNA (Fig 1B). However, the 2-fold decrease in GPR120 mRNA level in response to IL-1 β was less than with TNF α and there was no difference between the 4 and 24 h time-points. The effect of IL-1 β on *GPR84* expression was dramatic with the mRNA level being >500-fold higher at 4 h compared with the control cells. This considerable effect of IL-1 β was relatively transitory, with the increase in mRNA level falling sharply by 24 h to 15.7-fold relative to the controls. There was also a marked stimulation of *GPR41* expression in response to IL-1 β , the mRNA level being 16-fold and 37-fold higher than in the control cells at 4 and 24 h, respectively (Fig 1B).

Incubation with rosiglitazone led to a significant increase in *GPR120* gene expression at both 4 and 24 h of treatment. With the highest dose of rosiglitazone, there was a nearly 8-fold increase in GPR120 mRNA level at 24 h (Fig. 2A). *GPR41* expression was also stimulated by rosiglitazone, paralleling the response of *GPR120*; the high dose of the PPAR γ agonist resulted in a 10-fold increase in GPR41 mRNA level at 24 h (Fig. 2A). In contrast to *GPR120* and *GPR41*, rosiglitazone had no effect on *GPR84* expression (Fig. 2A).

Insulin had no significant effect on *GPR120* gene expression following 24 h of treatment, but there was a small stimulation with the higher dose at 4 h, mRNA level being increased 4.7-fold relative to the controls (Fig. 2B). There was no significant effect of insulin on either *GPR84* (Fig 2B) or *GPR41* expression (result not shown). Dexamethasone, with its anti-inflammatory action, was without effect on GPR120 mRNA level, irrespective of the dose or incubation time (Fig 2C). There was also no significant effect of the glucocorticoid on GPR84 mRNA, except at 24 h with the high dose where there was a small decrease (to 35% of the controls). There was also little effect of dexamethasone on *GPR41* expression, except for a small though statistically significant, increase in mRNA level at 4 h with the higher dose (result not shown).

In the next set of experiments, the effects of two PUFAs were examined. The adipocytes were incubated with two different concentration of the fatty acids, as in the other studies. There was no significant effect of linoleic acid, an *n*-6 PUFA, on *GPR120*, *GPR84* or *GPR41* expression at either 4 or 24 h with both of the concentrations employed (Fig 3A). DHA, an *n*-3 PUFA which is a natural ligand for GPR120 (Milligan *et al.*, 2015; Ulven & Christiansen, 2015; Calder, 2016), also had no significant effect on GPR84 or GPR41 mRNA level at 4 or 24 h, and nor was there any effect on GPR120 mRNA level at 4 h (Fig 3B). There was, however, a small (2.2-fold), but statistically significant, increase at 24 h with the higher concentration of DHA (Fig 3B).

The final agent examined was TUG891, a synthetic agonist to GPR120 (Milligan *et al.*, 2015; Gozal *et al.*, 2016). Treatment with TUG891 had no significant effect on GPR120 gene expression, nor on the expression of GPR84 and GPR41; this was the case at 4 and 24 h of treatment and with both low and high doses of the agonist (Fig. 3C)

Effect of TUG891 on the inflammatory response in adipocytes

In view of the strong down-regulation of *GPR120* expression by TNF α and IL-1 β , in the next studies the effect of GPR120 agonists on the inflammatory response evoked by these pro-inflammatory cytokines was examined. In the first experiment, adipocytes were treated with either TNF α or IL-1 β in the presence and absence of TUG891 for 4 h to assess the effect of the agonist on the acute response to the pro-inflammatory cytokines. PCR arrays containing probes for 84 cytokine and chemokine genes were used to examine the response to TNF α and whether this was attenuated by TUG891. The results in Table 1 indicate that TNF α had a powerful stimulatory effect on the expression of a substantial number of cytokine and chemokine genes (the full gene list is given in Appendix A). A total of 37 genes of those probed by the arrays exhibited increased expression (Appendix A); the mRNA level of 8 genes increased >100-fold with a further 10 exhibiting >10-fold increases (Table 1). The most strongly up-regulated genes were *CXCL10*, *CCL5* and *CCL1*, the mRNA levels of which were 907-, 438- and 395-fold

higher, respectively, than in the control untreated adipocytes. The expression of a small number of genes was inhibited by TNF α , with *IL11* and *IL16* (8.8-fold reduction in mRNA level) being the most strongly down-regulated (Table 1).

In contrast to TNF α , incubation with TUG891 had little effect on cytokine and chemokine expression (Table 1). *IL15* was the only gene for which there was a statistically significant reduction in mRNA level (to 68% of control cells). There were, however, significant increases in mRNA for 19 genes, but for all but two of these the increase in level was just 1.1 to 2-fold, with the highest (*TNFSF11*) being 3.5-fold. When the adipocytes were incubated with TNF α and TUG891 together, the changes in mRNA level relative to the untreated cells were very similar to those with TNF α alone, both in terms of the scale of change and the rank order of the responsive genes (Table 1). For example, as with TNF α on its own *CXCL10* and *CCL5* were the most strongly up-regulated genes with the combination of TNF α +TUG891, while *IL16* was the most strongly down-regulated.

The impact of TUG891 on the response to TNF α is demonstrated most clearly by comparing mRNA levels in the TNF α +TUG891 group compared with TNF α alone. Table 1 shows that the presence of TUG891 led to a reduction in the mRNA level for only three genes, there being no significant change for >70% of the genes probed by the arrays. The three genes where the TNF α -stimulated expression was significantly inhibited by TUG891 were *CXCL10*, *TGFB2* and *TNFSF10*, but in each case the decrease in the mRNA level was <30%; the fall in *CXCL10* mRNA level in the presence of TUG891 was just 18%. Paradoxically, rather than substantial decreases there was a statistically significant, albeit small, increase in mRNA level for 17 genes in the TUG891+TNF α group compared with TNF α alone; however, most of these changes resulted in increases of <1.5-fold with the highest (*IL1RN*) being 1.9-fold.

A similar study was conducted with adipocytes incubated with IL-1 β in the presence and absence of TUG891, and in view of the results with TNF α the expression of selected genes was examined rather than multiple genes through PCR arrays. While treatment with IL-1 β resulted in a substantial increase in *CCL2*, IL-1 β and IL-6 mRNA levels, the addition of TUG891 did not attenuate the response (Fig 4). Similarly, the down-regulation of *ADIPOQ* expression by IL-1 β was not modified by TUG891. The presence of TUG891 also did not attenuate the increase in *GPR84* expression stimulated by IL-1 β , but paradoxically it accentuated the reduction in *GPR120* mRNA level induced by the cytokine (Fig 5A).

Analysis of selected inflammation-related adipokines in the medium showed that the presence of TUG891 did not alter the level of the secreted proteins evident with IL-1 β alone; there was no difference between the IL-1 β +TUG891 and IL-1 β groups in the amounts of IL-6,

IL-16, MCP-1 or adiponectin in the medium (Fig 6A). Similarly, with the adipocytes treated with TNF α , the presence of TUG891 did not alter the quantity of MCP-1, IL-16 and adiponectin detected in the medium. In the case of TNF α there was actually a small, but statistically significant, increase in the amount of this adipokine released by cells incubated with TUG891+TNF α relative to those with TNF α alone (Fig 6B).

Effect of DHA on IL-1 β induced changes in gene expression

In the final experiment, adipocytes were incubated with DHA as a natural ligand to GPR120, and the inflammatory response induced by IL-1 β examined in the presence and absence of the fatty acid. The adipocytes were incubated for 4 and 24 h in order to assess whether any putative effects are acute or chronic; results are, however, shown for 24 h only as no acute effects were evident.

The presence of DHA did not lead to an attenuation of the increases in IL-6 and IL-1 β mRNA level induced by IL-1 β at either 4 or 24 h (Fig 7). There was, however, a significant attenuation of *CCL2* gene expression at 24 h (Fig 7). Similarly, at 24 h the presence of DHA resulted in an attenuation in the decrease in *IL16* and *ADIPOQ* expression observed with IL-1 β (Fig 7). Thus, some modest effects of DHA on the inflammatory response induced by the pro-inflammatory cytokine are apparent. However, the presence of DHA did not have a protective effect on the reduction in *GPR120* gene expression occurring with IL-1 β (Fig 7). There was, in contrast, an attenuation by DHA of the IL-1 β -induced increase in *GPR84* mRNA level (Fig 7).

As in the studies with TUG891, the effect of DHA on the release of key inflammation-related adipokines from adipocytes treated with IL-1 β was examined. The 24 h samples were used in order to assess the consequences of longer-term exposure to the fatty acid and the cytokine. The presence of DHA did not result in any changes in the amount of IL-6, IL-16, MCP-1 or adiponectin found in the medium (Fig 6C).

Discussion

The present study demonstrates that several factors influence the expression of the genes encoding the major fatty acid receptors/sensors - GPR120, GPR41 and GPR84 - in human adipocytes. Major responses were observed following exposure to pro-inflammatory mediators, while insulin, the glucocorticoid dexamethasone, and the fatty acids DHA and linoleic acid had no, or limited, effects. In a previous report it was noted that incubation of human adipocytes with macrophage-conditioned medium (from U937 cells) resulted in a substantial stimulation of *GPR84* expression, while *GPR120* expression was markedly inhibited (Trayhurn & Denyer, 2012). A substantial up-regulation of *GPR84* expression has also been observed in murine 3T3-L1 adipocytes co-cultured with the RAW264 macrophage cell line (Nagasaki et al., 2012).

Macrophage-conditioned medium contains a multiplicity of secretory products from the activated immune cells, among the most important of which are the cytokines TNF α and IL-1 β . *GPR120* gene expression was inhibited in the present study by both these cytokines, the reduction in mRNA level being particularly marked with TNF α . In contrast, there was a major stimulation of *GPR84*, and to a lesser extent *GPR41*, expression by both TNF α and IL-1 β . Stimulation of *GPR84* expression in 3T3-L1 and human adipocytes (adipose-derived stromal cells) by TNF α has been observed previously, and also in response to lipopolysaccharide (Nagasaki et al., 2012). In the present study, the stimulatory effect on *GPR84* expression was greatest with IL-1 β at 4 h, but there was no difference between IL-1 β and TNF α in the peak effect on *GPR41* expression. Differences in the time-course of the response of the two genes were apparent, however, with that of *GPR84* being greatest at 4 h, with the mRNA level falling sharply by 24 h; this effect was particularly evident with IL-1 β . In the case of *GPR41*, gene expression was more strongly stimulated at 24 h, and this was most apparent with TNF α .

Although a signal for *GPR41* was consistently observed in the present work using real-time PCR, the mRNA was not detected in the study on macrophage-conditioned medium which utilised human Oligo microarrays (O'Hara et al., 2009). Studies on murine adipose tissue have also reported a lack of *GPR41* expression (Zaibi et al., 2010), although some other reports have observed clear signals together with functional responses associated with this receptor (Xiong *et al.*, 2004; Han *et al.*, 2014). GPR41, for which the main ligands are short chain fatty acids (Milligan *et al.*, 2014; Ulven & Christiansen, 2015), is less well-characterised than the other GPCR fatty acid receptors, but it was reported to mediate the stimulation of leptin secretion from adipocytes by propionate and butyrate (Xiong et al., 2004). Subsequent studies have suggested, however, that this role is played by GPR43 rather than GPR41 (Zaibi et al., 2010).

The individual effects of IL-1 β and TNF α suggest that these cytokines are key factors in the responses of human adipocytes to macrophage-conditioned medium (Trayhurn & Denyer, 2012), although the involvement of other macrophage secretions cannot be excluded. The inflammatory response appears to be central to the overall regulation of the expression of GPCR fatty acid receptor genes, at least in adipocytes, and in the function of the encoded receptors. The up-regulation of *GPR120* expression by rosiglitazone is also of note in this regard, given the anti-inflammatory actions associated with the PPAR γ receptor.

GPR84, the primary ligands for which are medium chain fatty acids of carbon chain length 9-14 (Wang et al., 2006), has been proposed as a pro-inflammatory receptor (Suzuki et al., 2013). For example, activation of GPR84 is reported to amplify the production of IL-8 and IL-12 p40 by lipopolysaccharide in polymorphonuclear leukocytes as well as the production of TNF α in

macrophages (Wang *et al.*, 2006; Suzuki *et al.*, 2013). On this basis, the stimulation of GPR84 synthesis in human adipocytes by cytokines, derived either from macrophages or from other adipocytes, would be predicted to result in an amplification of the inflammatory response in adipose tissue.

GPR120 has been the focus of considerable interest following the report that it is a receptor for *n*-3 PUFAs and mediates their anti-inflammatory and insulin-sensitising actions (Oh *et al.*, 2010; Oh & Olefsky, 2012). Medium-chain fatty acids are also ligands for this receptor. GPR120 has been implicated in energy balance, loss or dysfunction of the receptor leading to obesity in both mice and humans as well as to glucose intolerance and adipose tissue inflammation (Ichimura *et al.*, 2012). Other functions in which the receptor is implicated include the promotion of GLP-1 secretion from gastrointestinal L- and K- cells, and the regulation of adipogenesis (Gotoh *et al.*, 2007; Moniri, 2016). The proposed insulin-sensitising and anti-inflammatory actions of GPR120 are of particular significance. GPR120 activation enhances glucose uptake and the translocation of GLUT4 in 3T3-L1 adipocytes, while *in vivo* studies have demonstrated that diets enriched in *n*-3 PUFAs lead to a reduction in macrophage infiltration in white adipose tissue of mice together with a reduction in the expression of pro-inflammatory genes such as *IL1B*, *IL6* and *CCL2*, effects that are attenuated in GPR120 knockout mice (Oh *et al.*, 2010).

As a consequence of its apparent regulatory function in inflammation and insulin sensitivity, GPR120 has been viewed as a therapeutic target in the treatment of insulin resistance, type 2 diabetes and the metabolic syndrome (Milligan *et al.*, 2015; Ulven & Christiansen, 2015). Natural ligands for the receptor include eicosapentanoic acid, pinolenic acid and DHA (Christiansen *et al.*, 2015), while TUG891 and NCG21 are current examples of synthetic ligands (Hudson *et al.*, 2013; Milligan *et al.*, 2015; Ulven & Christiansen, 2015). In view of the anti-inflammatory action associated with GPR120 it appears paradoxical that macrophage secretions, and specifically IL-1 β and TNF α , lead to an inhibition of its synthesis. This is based on the marked inhibitory effects on gene expression; attempts to measure GPR120 protein in human adipocytes by Western blotting were unsuccessful (which may reflect low levels in the cells). A question of significance is whether the anti-inflammatory actions of *n*-3 PUFAs are compromised, or attenuated, by a loss in GPR120 receptor production under inflammatory conditions?

In the present study, the presence of TUG891 did not inhibit the acute stimulation of the expression of cytokine and chemokine genes induced by TNF α , where PCR arrays were employed, or by IL-1 β when selective genes were examined. The only gene whose expression was

strongly stimulated by $\text{TNF}\alpha$ and which exhibited an inhibitory response to the simultaneous presence of TUG891 was *CXCL10*, which encodes the interferon gamma-induced protein 10 (IP-10), and even this effect was modest. Thus, there was no evidence of a significant anti-inflammatory effect of the GPR120 ligand in human adipocytes. Previous studies with TUG891 have indicated that it mimics the effects of GPR120 activation, such as the enhancement of glucose uptake by 3T3-L1 cells and inhibition of the release of pro-inflammatory mediators in macrophage cell lines (Hudson et al., 2013). In addition, TUG891 has recently been shown to significantly reduce inflammation and insulin resistance induced by chronic sleep fragmentation in mice (Gozal et al., 2016); the effects on inflammation were, however, based on macrophages present in the stromal-vascular fraction of white fat rather than the adipocyte component of the tissue.

DHA, a natural ligand for GPR120, did have some selective, though limited, effect on the response of human adipocytes to $\text{IL-1}\beta$ with prolonged incubation. While DHA did not modify the $\text{IL-1}\beta$ induced stimulation of *IL6* and *IL1B* expression, there was a distinct attenuation of *CCL2* expression, indicating that the production of a major chemoattractant (MCP-1) was reduced by the GPR120 ligand (in contrast to TUG891). Interestingly, a reversal of the fall in *ADIPOQ* expression induced by $\text{IL-1}\beta$ was also evident in the presence of DHA, implying that the *n-3* PUFA aided the preservation of adiponectin synthesis. This would be consistent with the anti-inflammatory effect of DHA given that adiponectin itself has an anti-inflammatory (Ouchi et al., 1999; Yokota et al., 2000), as well as insulin-sensitising, action (Berg et al., 2001; Yamauchi et al., 2001). However, DHA did not alter the suppressive effect of $\text{IL-1}\beta$ on the secretion of adiponectin into the medium, nor that of IL-6 and IL-16 . The amount of these adipokines measured in the medium relates, of course, to the total released over the full 24 h incubation period, while the measurements of mRNA level reflect the situation pertaining at the 24 h time-point itself.

The absence of a strong anti-inflammatory effect of both TUG891 and DHA in human adipocytes exposed to $\text{IL-1}\beta$ and $\text{TNF}\alpha$ is not consistent with the proposition that *n-3* PUFAs inhibit inflammation (Calder, 2011; Calder, 2015) and that GPR120 is a key receptor in mediating this action (Oh et al., 2010; Oh & Olefsky, 2012). It is possible, of course, that human adipocytes are less responsive to both natural and synthetic GPR120 ligands than other cell types, particularly macrophages. If this is the case, then one potential explanation is that the amount of GPR120 is low in human adipocytes, and as noted above we were unable to successfully measure the protein in the human fat cells employed.

An intriguing possibility is that the inflammatory response within human adipocytes is not significantly attenuated by DHA and other GPR120 receptor ligands since pro-inflammatory cytokines, as well as other macrophage- and adipocyte-derived factors, induce a substantial down-regulation of the synthesis of this receptor. The presence of DHA did not prevent the inhibition of *GPR120* gene expression induced by IL-1 β , so the apparent detrimental effects of an inflammatory stimulus on the production of the receptor are not ameliorated by the *n*-3 PUFA. Furthermore, the combination of TUG891 and IL-1 β resulted in a greater down-regulation of *GPR120* expression than with IL-1 β alone. DHA (though not TUG891) did, however, attenuate the increase in *GPR84* expression induced by IL-1 β , and since this receptor is considered pro-inflammatory (Suzuki et al., 2013) the *n*-3 PUFA did in effect exhibit some anti-inflammatory action.

Conclusions

The regulation of *GPR120* and *GPR84* expression in human adipocytes is strongly influenced by major pro-inflammatory cytokines, with an inhibition of the former and stimulation of the latter. In human fat cells, neither a natural nor a synthetic ligand of GPR120 was able to attenuate the major inflammatory response stimulated by TNF α or IL-1 β , and this may in part be a consequence of an inflammation-induced reduction in the expression of the receptor. Adipocytes are able to exhibit a substantial inflammatory response (Rajala & Scherer, 2003; Trayhurn & Wood, 2004; Trayhurn, 2005), and despite this not being significantly attenuated by GPR120 agonists, *n*-3 PUFAs may have an overall anti-inflammatory action in adipose tissue through the other cell types within the tissue, especially the macrophages that are recruited in obesity.

Declaration of interest

We thank the Distinguished Scientist Fellowship Programme, King Saud University, for financial support of this work. LM is grateful to Professor Banni, University of Cagliari, for his support of her Training Fellowship at the University of Buckingham. We thank Professor Trond Ulven, University of Southern Denmark, for the provision of TUG891. The authors declare that they have no conflicts of interest with regard to this study.

Appendix A. Supplementary data

Supplementary data related to this article is attached.

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Table 1. Effect of TNF α and the GPR120 agonist TUG891 on the expression of cytokine and chemokines genes in human adipocytes assessed by PCR arrays.

Gene	TNF α cpd to control (fold-change)	TUG891 cpd to control (fold-change)	TNF α +TUG891 cpd to control (fold-change)	TNF α +TUG891 cpd to TNF α (fold-change)
<i>CXCL10</i>	907***	0.285	747***	0.823*
<i>CCL5</i>	438***	0.968	461***	1.05
<i>CCL1</i>	395***	1.23	365***	0.922
<i>CXCL11</i>	305***	0.441	267***	0.875
<i>CSF2</i>	253***	2.47*	378***	1.49***
<i>CCL3</i>	207***	0.619	279***	1.35
<i>CCL20</i>	157***	1.92***	194***	1.24*
<i>CX3CL1</i>	140***	1.33	121***	0.863
<i>CSF3</i>	93.7***	1.66	135***	1.44*
<i>CXCL8</i>	89.2***	1.37**	98.8***	1.11*
<i>TNF</i>	83.9***	0.695	69.3***	0.826
<i>CCL19</i>	59.6***	0.143	42.2***	0.708
<i>IL1B</i>	55.9***	0.690	63.0***	1.13
<i>CCL2</i>	30.8***	1.13	31.9***	1.04
<i>CXCL2</i>	29.1***	1.41***	30.6***	1.05
<i>CXCL1</i>	25.3***	1.40**	28.0***	1.11**
<i>CCL7</i>	16.8***	1.73***	23.3***	1.39**
<i>IL6</i>	11.0***	1.78***	14.9***	1.36**
<i>LIF</i>	8.62***	1.86**	13.7***	1.59**
<i>BMP2</i>	8.24***	2.31**	14.7***	1.79**
<i>IL15</i>	6.69***	0.682**	6.74***	1.01
<i>CXCL5</i>	6.64***	1.02	8.09***	1.22**
<i>TNFRSF11B</i>	3.46***	1.39**	3.78***	1.09
<i>TNFSF11</i>	3.41	3.54*	14.3	4.18
<i>IL1RN</i>	3.14***	1.93**	6.03***	1.92**
<i>TGFB2</i>	2.37***	1.13	1.84***	0.779**
<i>TNFSF10</i>	1.36**	1.02	0.973	0.713***
<i>TNFSF13B</i>	1.36***	0.985	1.22**	0.896
<i>IL12A</i>	0.806*	1.37**	1.08	1.34**
<i>ADIPOQ</i>	0.729*	0.987	0.839	1.15
<i>SPP1</i>	0.675***	1.03	0.796*	1.18**
<i>BMP6</i>	0.552***	1.23**	0.696***	1.26*
<i>IL11</i>	0.226*	1.89*	0.670	2.97
<i>IL16</i>	0.113***	1.09	0.246***	2.18

Differentiated human adipocytes were incubated in the presence or absence of human recombinant TNF α (100 ng/ml), with and without TUG891 (10 μ M), for 4 h. The expression of 84 cytokine and chemokine genes was probed using PCR pathway arrays. The results, which are for selected genes (the full gene list is given in Appendix A) are expressed as fold-changes in mRNA level for each treatment relative to the group to which it is compared, and are the means of 6 sets of adipocytes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control or to TNF α treated cells. All other differences are not statistically significant ($P > 0.05$).

Legends to Figures

Figure 1. Effect of (A) TNF α and (B) IL-1 β on the expression of the *GPR120*, *GPR84* and *GPR41* genes in human adipocytes. The adipocytes were incubated with TNF α or IL-1 β for either 4 or 24 h; LD, low dose TNF α (5 ng/ml) and HD, high dose TNF α (100 ng/ml). IL-1 β was used at a concentration of 2 ng/ml. The results, which are expressed as fold-changes in mRNA level in cells treated with TNF α or IL-1 β relative to the respective control cells, are means \pm SE (bars) for 4-6 sets of adipocytes. ** $P < 0.01$, *** $P < 0.001$ compared with the control cells (Con) at the same time point.

Figure 2. Effect of (A) rosiglitazone, (B) insulin and (C) dexamethasone on the expression of the *GPR120* and *GPR84* genes in human adipocytes. Data for *GPR41* are also shown in the case of rosiglitazone. The adipocytes were incubated with rosiglitazone (LD, low dose = 0.1 μ M and HD, high dose = 1 μ M), insulin (LD, low dose = 1 nM; HD, high dose = 20 nM), or dexamethasone (LD, low dose = 2 nM; HD, high dose = 20 nM) for either 4 or 24 h. The results, which are expressed as fold-changes mRNA level in the cells treated with each agent relative to the respective control cells, are means \pm SE (bars) for 2-4 sets of adipocytes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control cells (Con) at the same time point.

Figure 3. Effect of (A) linoleic acid, (B) docosahexaenoic acid and (C) TUG891 on the expression of the *GPR120* and *GPR84* genes in human adipocytes. The adipocytes were incubated with linoleic acid (LD, low dose = 50 μ M; HD, high dose = 200 μ M) or docosahexaenoic acid (LD, low dose = 25 μ M; HD, high dose = 100 μ M), or TUG891 (LD, low dose = 500 nM; HD, high dose = 10 μ M) for either 4 or 24 h. The results, which are expressed as fold-changes in mRNA level in the cells treated with the fatty acid or TUG891 relative to the control cells (Con), are means \pm SE (bars) for 2-4 sets of adipocytes. * $P < 0.05$ compared with the control cells at the same time point.

Figure 4. Effect of TUG891 on the changes induced by IL-1 β in the expression of the *CCL2*, *IL1B*, *IL6* and *ADIPOQ* genes in human adipocytes. The adipocytes were incubated with IL-1 β (0.5 ng/ml), with and without TUG891 (10 μ M), for 4 h. mRNA levels were measured by real-time PCR. The results, which are expressed as fold-changes in the mRNA level in treated cells relative to the control cells (Con), are means \pm SE (bars) for 4-6 sets of adipocytes. *** $P < 0.001$ compared with the control cells.

Figure 5. Effect of (A) TUG891 and (B) docosahexaenoic acid on the changes induced by IL-1 β in the expression of the GPR120 and GPR84 genes in human adipocytes. The adipocytes were incubated with IL-1 β (0.5 ng/ml), with and without TUG891 (10 μ M) for 4 h, or with and without docosahexaenoic acid (100 μ M), for 24 h. The results, which are expressed as fold-changes in the treated cells relative to the control cells (Con), are means \pm SE (bars) for 4-6 sets of adipocytes. * P <0.05, ** P <0.01, *** P <0.001 compared with the control cells. ## P <0.01, ### P <0.001 compared with TUG891 alone; # P <0.05 compared with DHA alone; \surd P <0.01 compared with IL-1 β alone.

Figure 6. Release of inflammation-related adipokines from human adipocytes following treatment with either IL-1 β or TNF α in the presence and absence of a GPR120 agonist. (A) IL-1 β (0.5 ng/ml) \pm TUG891 (10 μ M), (B) TNF α (100 ng/ml) \pm TUG891 (10 μ M) (C) IL-1 β \pm docosahexaenoic acid (DHA, 100 μ M). The medium was from the adipocytes used in Figs 6, 7 and 9, and Table 1. The results are means \pm SE (bars) for 4 sets of adipocytes in each group; open bars, cytokine alone; filled bars cytokine + TUG891 or DHA. *** P <0.001 compared with the cells incubated without TUG891 (open bars).

Figure 7. Effect of docosahexaenoic (DHA) acid on the changes induced by IL-1 β in the expression of *CCL2*, *IL1B*, *IL6* and *ADIPOQ* in human adipocytes. The adipocytes were incubated with IL-1 β (0.5 ng/ml), with and without DHA (100 μ M), for 24 h. The results, which are expressed as fold-changes in mRNA level in treated cells relative to the control cells (Con), are means \pm SE (bars) for 6 sets of adipocytes. *** P <0.001 compared with the control cells. # P <0.05, ## P <0.01, ### P <0.001 compared with DHA alone.

Figure 1

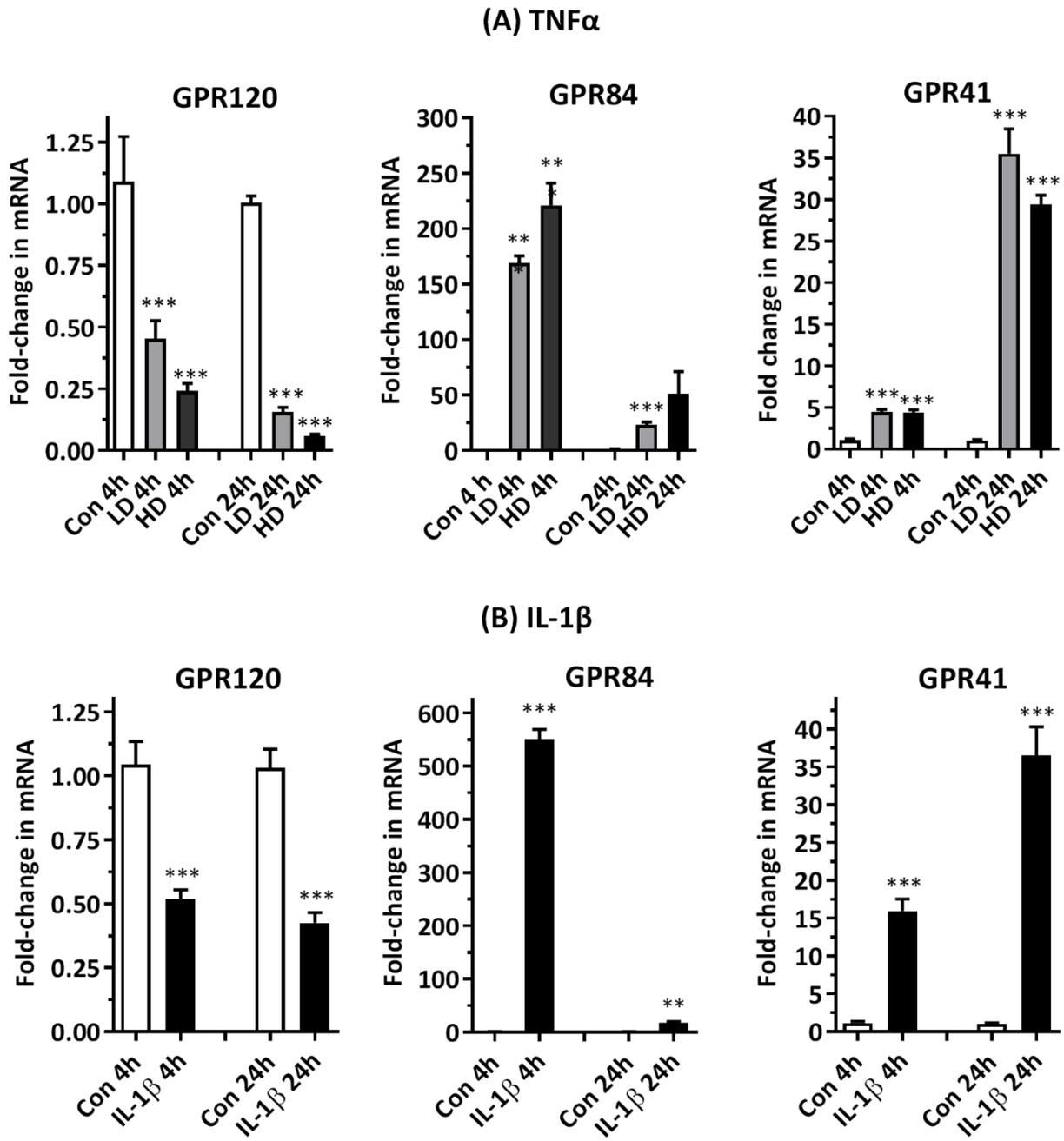
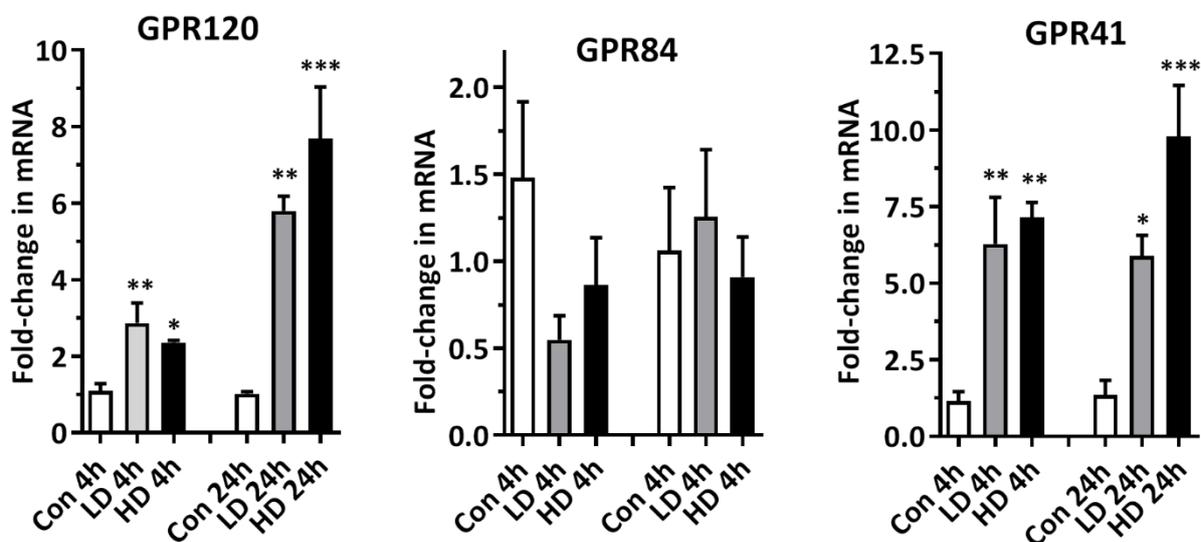
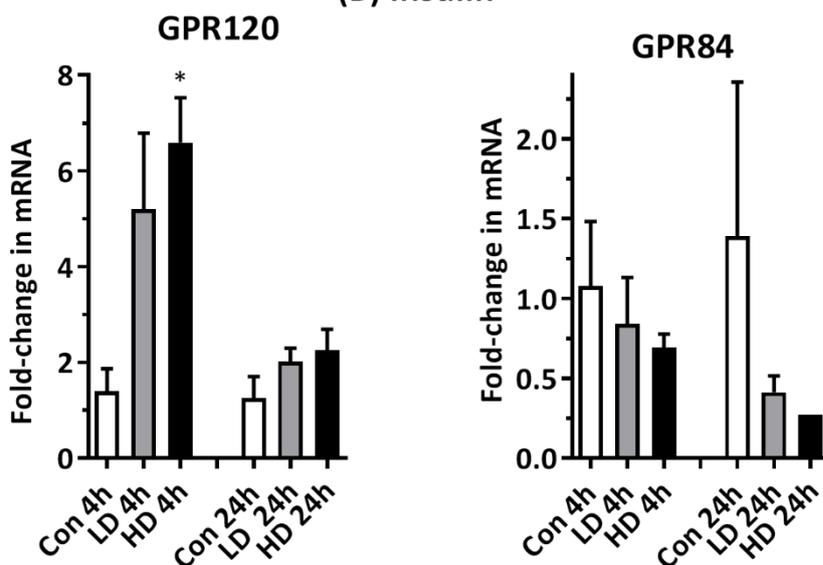


Figure 2

(A) Rosiglitazone



(B) Insulin



(C) Dexamethasone

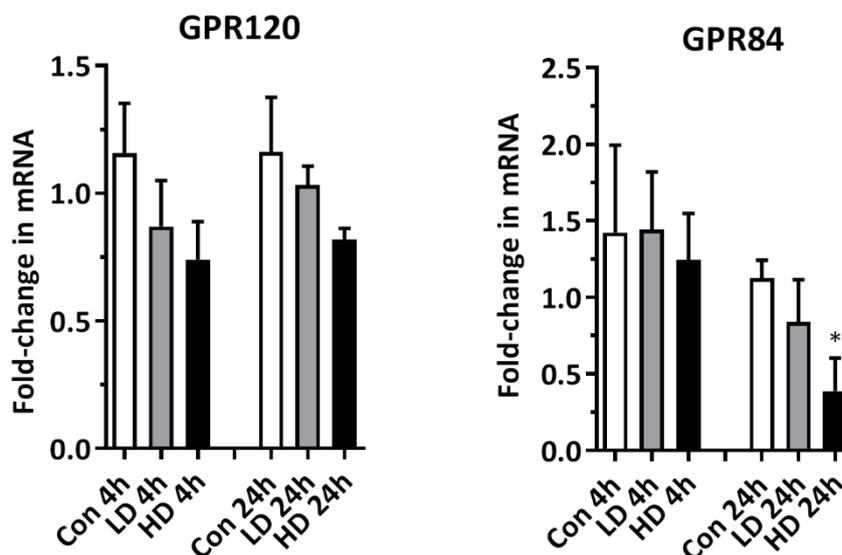
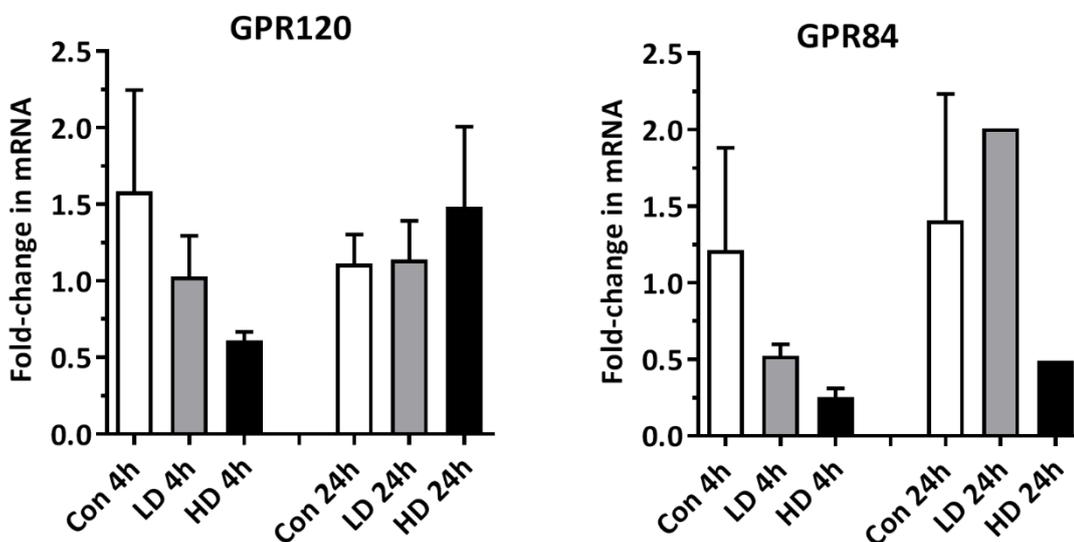
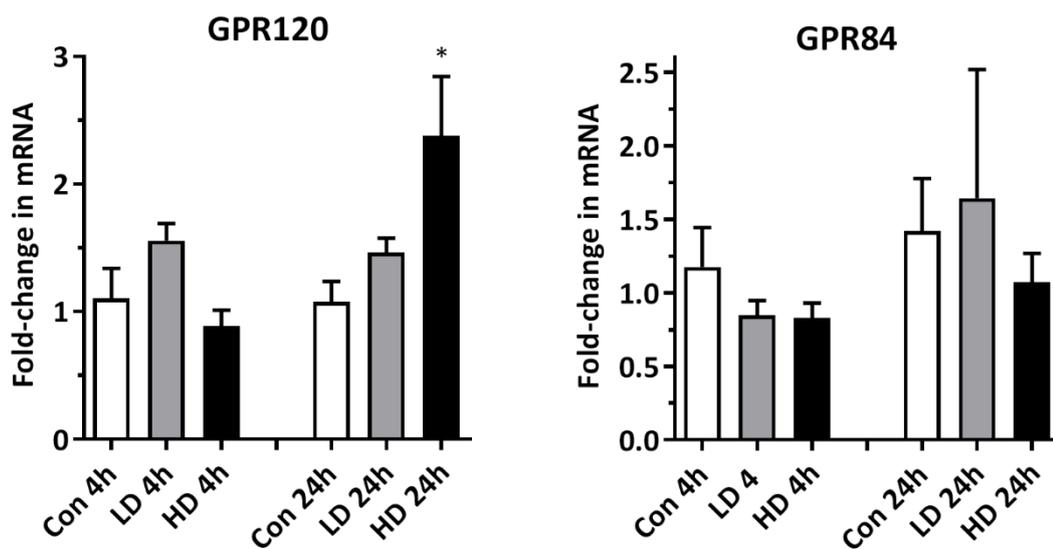


Figure 3

(A) Linoleic acid



(B) Docosaehaenoic acid



(C) TUG891

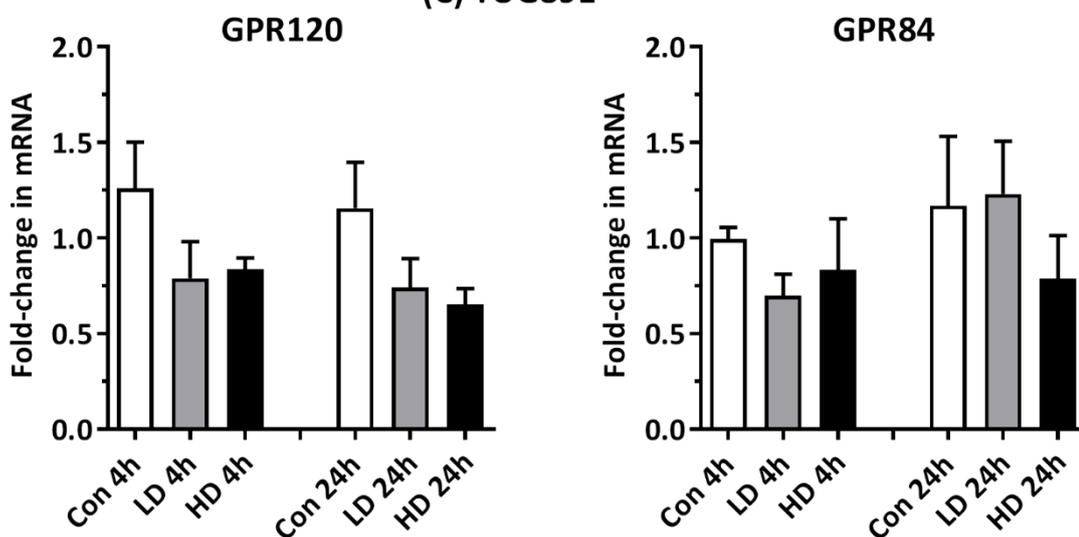


Figure 4

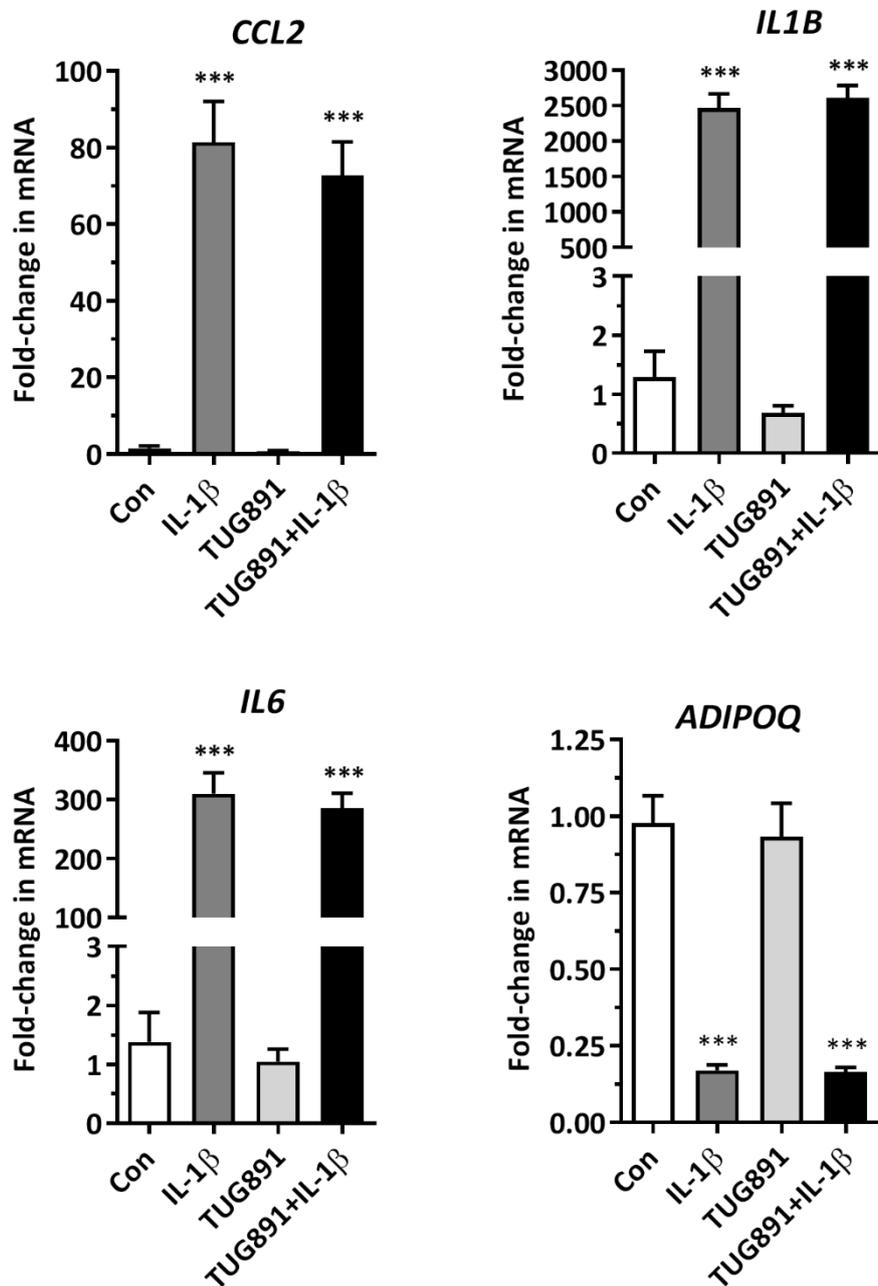


Figure 5

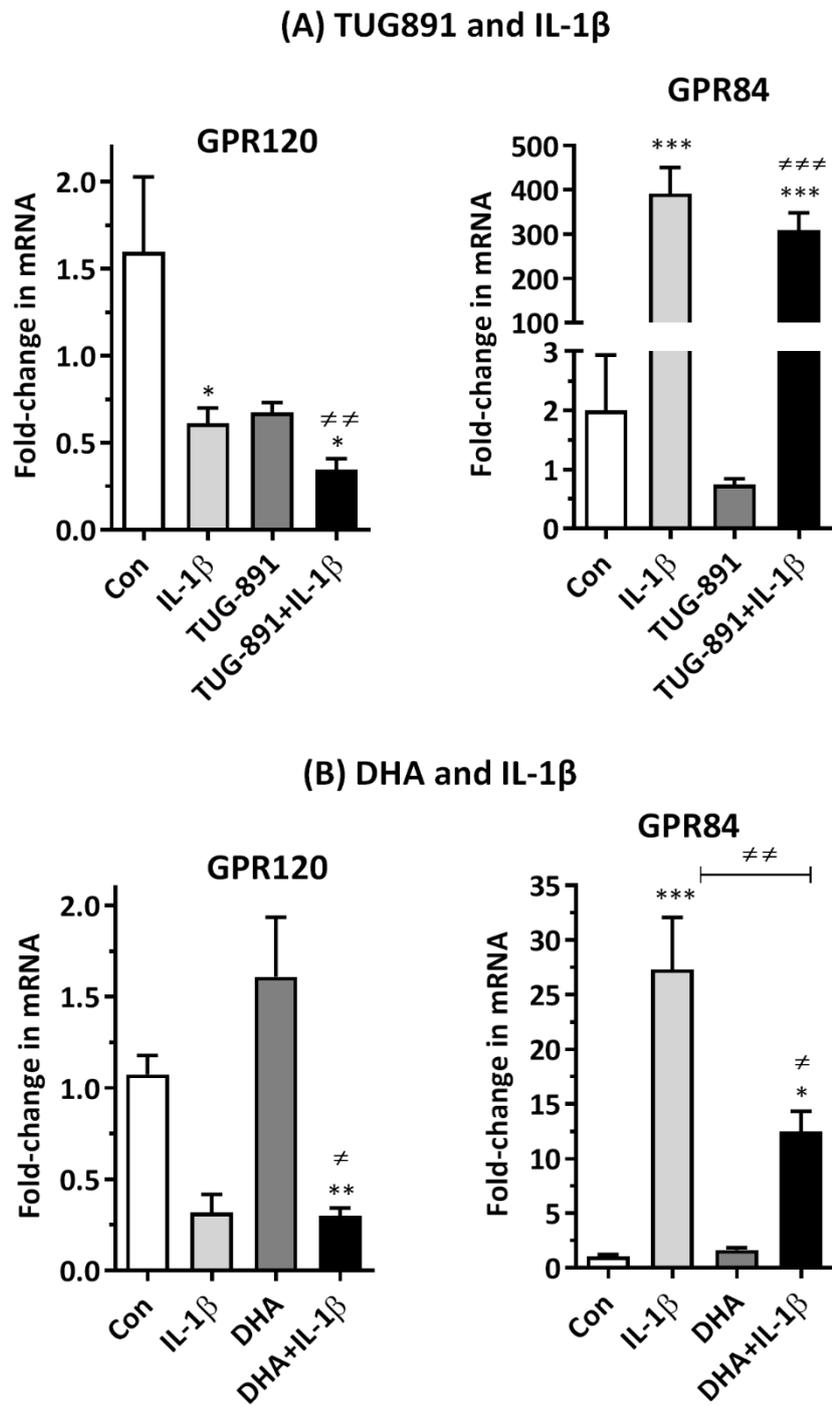


Figure 6

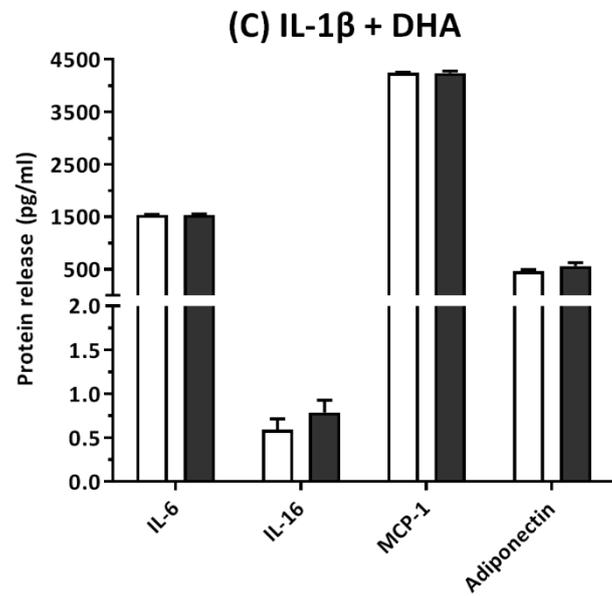
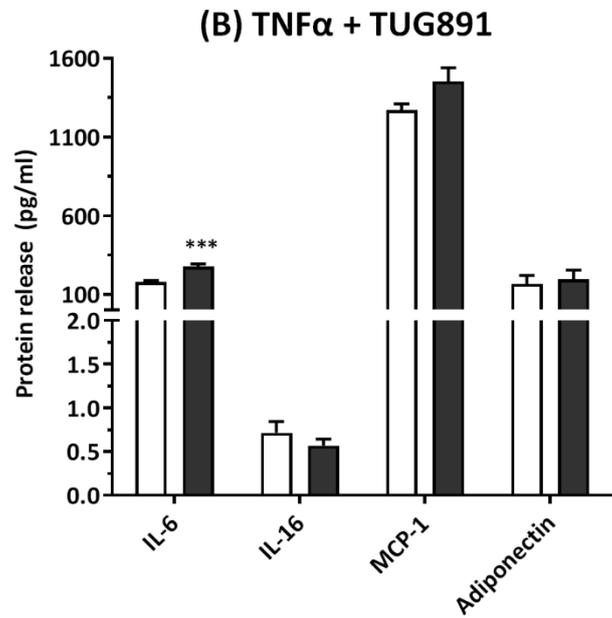
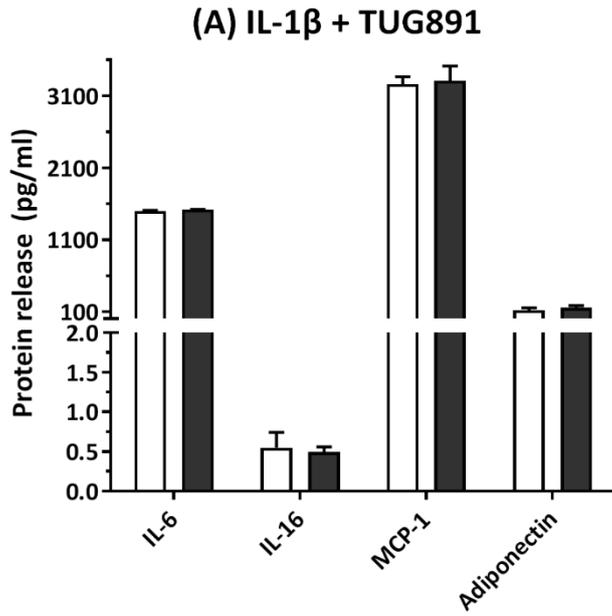
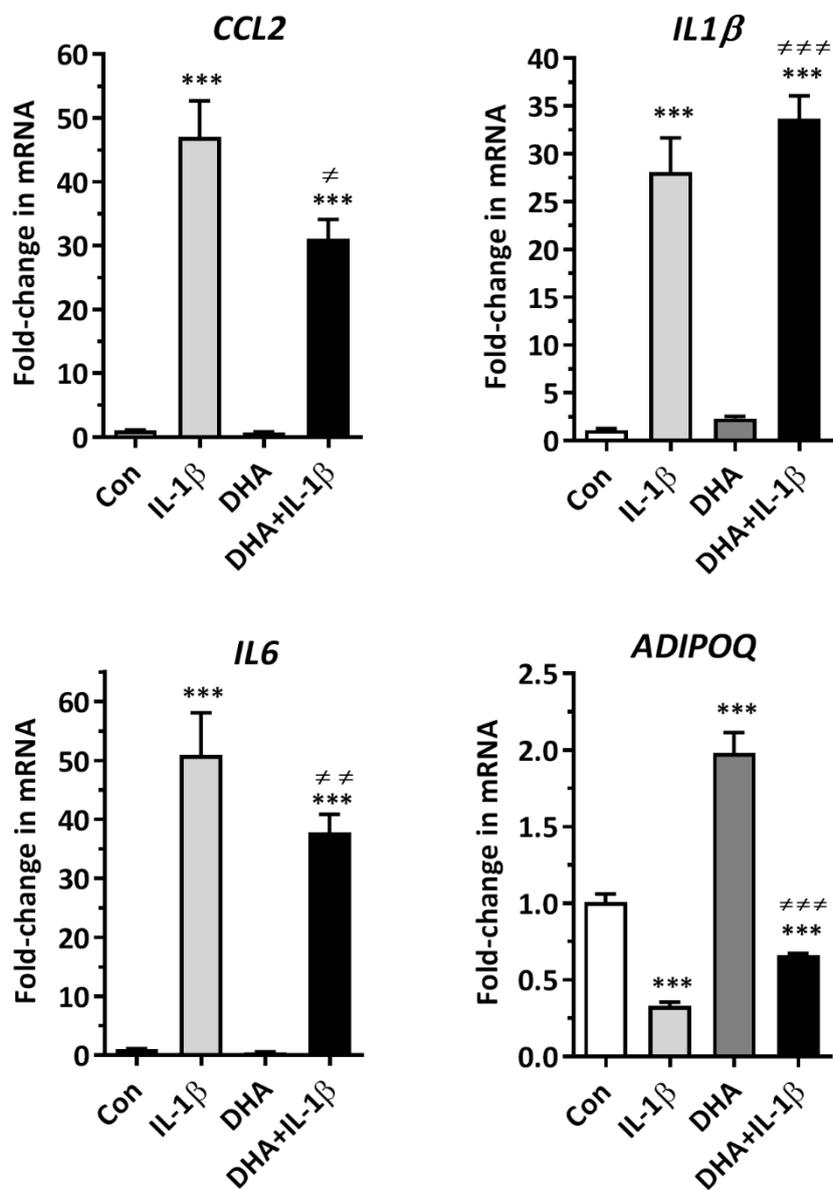


Figure 7



Appendix A
Supplement 1

Table 1. Effect of TNF α and the GPR120 agonist TUG891 on the expression of cytokine and chemokines genes in human adipocytes assessed by PCR arrays.

Gene	TNF α cpd to control (fold-change)	TUG891 cpd to control (fold-change)	TNF α +TUG891 cpd to control (fold-change)	TNF α +TUG891 cpd to TNF α (fold-change)
<i>CXCL10</i>	907***	0.285	747***	0.823*
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<i>CCL1</i>	395***	1.23	365***	0.922
<i>CXCL11</i>	305***	0.441	267***	0.875
<i>CSF2</i>	253***	2.47*	378***	1.49***
<i>CCL3</i>	207***	0.619	279***	1.35
<i>CCL20</i>	157***	1.92***	194***	1.24*
<i>CX3CL1</i>	140***	1.33	121***	0.863
<i>CSF3</i>	93.7***	1.66	135***	1.44*
<i>CXCL8</i>	89.2***	1.37**	98.8***	1.11*
<i>TNF</i>	83.9***	0.695	69.3***	0.826
<i>CCL19</i>	59.6***	0.143	42.2***	0.708
<i>IL1B</i>	55.9***	0.690	63.0***	1.13
<i>CCL2</i>	30.8***	1.13	31.9***	1.04
<i>CXCL2</i>	29.1***	1.41***	30.6***	1.05
<i>CXCL1</i>	25.3***	1.40**	28.0***	1.11**
<i>CCL7</i>	16.8***	1.73***	23.3***	1.39**
<i>IL6</i>	11.0***	1.78***	14.9***	1.36**
<i>CCL11</i>	9.63***	0.321	9.44**	0.981
<i>LIF</i>	8.62***	1.86**	13.7***	1.59**
<i>BMP2</i>	8.24***	2.31**	14.7***	1.79**
<i>IL15</i>	6.69***	0.682**	6.74***	1.01
<i>CXCL5</i>	6.64***	1.02	8.09***	1.22**
<i>CXCL9</i>	6.46*	1.03	3.40	0.527
<i>CSF1</i>	4.84***	1.09**	4.87***	1.01
<i>LTB</i>	3.91*	0.960	2.28	0.583
<i>TNFRSF11B</i>	3.46***	1.39**	3.78***	1.09
<i>TNFSF11</i>	3.41	3.54*	14.3	4.18
<i>IL1RN</i>	3.14***	1.93**	6.03***	1.92**
<i>TGFB2</i>	2.37***	1.13	1.84***	0.779**
<i>IL23A</i>	2.01***	1.82*	2.11**	1.05
<i>IL5</i>	1.90	1.14	0.237	0.124
<i>CCL13</i>	1.47***	0.958	1.79***	1.22*
<i>CNTF</i>	1.37***	1.21	1.52***	1.11
<i>BMP4</i>	1.37	1.14	1.82*	1.33
<i>TNFSF10</i>	1.36**	1.02	0.973	0.713***
<i>VEGFA</i>	1.36**	1.29**	1.68***	1.23**
<i>TNFSF13B</i>	1.36***	0.985	1.22**	0.896
<i>IL7</i>	1.36***	0.849	1.43***	1.05
<i>PPBP</i>	1.35	1.16	1.10	0.817
<i>CXCL16</i>	1.31*	1.22	1.24*	0.951

LTA	1.19	1.12	3.31*	2.79
IL18	1.08	0.837	1.12	1.04
MIF	1.01	1.07**	0.960	0.946
CXCL12	1.00	1.04	1.07	1.06
GPI	0.964*	1.02	0.990	1.03
CCL21	0.842	1.37	2.25	2.68
C5	0.825*	0.886	0.770**	0.933
IL27	0.808	1.68	1.58	1.95
IL12A	0.806*	1.37**	1.08	1.34**
NODAL	0.797	1.49	1.19	1.49
IL13	0.754	2.39	1.41	1.87
ADIPOQ	0.729*	0.987	0.839	1.15
MSTN	0.701	0.939	0.729	1.04
IL22	0.699*	0.995	1.24	1.77
SPP1	0.675***	1.03	0.796*	1.18**
THPO	0.658	1.21	0.999	1.52*
IL4	0.656	0.919	1.59	2.42
IL21	0.643	ND	0.790	1.23
BMP6	0.552***	1.23**	0.696***	1.26*
IL24	0.515	0.715	0.553	1.08
IL17F	0.445	0.554	1.08	2.42
BMP7	0.432	0.394	0.630	1.46
CCL18	0.419*	0.696	0.743	1.77
IFNA2	0.405	0.606	1.35	3.32
CD40LG	0.370	0.880	0.298	0.805
IL11	0.226*	1.89*	0.670	2.97
IL16	0.113***	1.09	0.246***	2.18

Differentiated human adipocytes were incubated in the presence or absence of human recombinant TNF α (100 ng/ml), with and without TUG891 (10 μ M), for 4 h. The expression of 84 cytokine and chemokine genes was probed using PCR pathway arrays. The results are expressed as fold-changes in mRNA level for each treatment relative to the group to which it is compared, and are the means of 6 sets of adipocytes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control or to TNF α treated cells. All other differences are not statistically significant ($P > 0.05$).

Annex: Acceptance Information

01-Aug-2017

Dear Professor Trayhurn:

Ref: IL-1 β and TNF α inhibit GPR120 (*FFAR4*) and stimulate GPR84 (*EX33*) and GPR41 (*FFAR3*) fatty acid receptor expression in human adipocytes: implications for the anti-inflammatory action of n-3 fatty acids

Our referees have now considered your paper and have recommended publication in Archives Of Physiology And Biochemistry. We are pleased to accept your paper in its current form which will now be forwarded to the publisher for copy editing and typesetting. The reviewer comments are included at the bottom of this letter.

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Sincerely,

Dr Eckel

Editor in Chief, Archives Of Physiology And Biochemistry

eckel@uni-duesseldorf.de