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Effects of AF3442 [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-(trifluoromethyl)benzamide], a novel inhibitor of human microsomal prostaglandin E synthase-1, on prostanoid biosynthesis in human monocytes in vitro

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ABSTRACT

Inhibitors of microsomal prostaglandin (PG) E synthase-1 (mPGES-1) are being developed for the relief of pain. Redirection of the PGH_2 substrate to other PG synthases, found both in vitro and in vivo, in mPGES-1 knockout mice, may influence their efficacy and safety. We characterized the contribution of mPGES-1 to PGH_2 metabolism in lipopolysaccharide (LPS)-stimulated isolated human monocytes and whole blood by studying the synthesis of prostanoids [PGE_2, thromboxane (TX)B_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$] and expression of cyclooxygenase (COX)-isozymes and down-stream synthases in the presence of pharmacological inhibition by the novel mPGES-1 inhibitor AF3442 [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-(trifluoromethyl)benzamide]. AF3442 caused a concentration-dependent inhibition of PGE_2 in human recombinant mPGES-1 with an IC_{50} of 0.06 μM. In LPS-stimulated monocytes, AF3442 caused a concentration-dependent reduction of PGE_2 biosynthesis with an IC_{50} of 0.41 μM. At 1 μM, AF3442 caused maximal selective inhibitory effect of PGE_2 biosynthesis by 61 ± 3.3% (mean ± SEM, $P < 0.01$ versus DMSO vehicle) without significantly affecting other prostanoids (i.e. TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$). In LPS-stimulated whole blood, AF3442 inhibited in a concentration-dependent fashion inducible PGE_2 biosynthesis with an IC_{50} of 29 μM. A statistically significant inhibition of mPGES-1 activity was detected at 10 and 100 μM (38 ± 14%, $P < 0.05$, and 69 ± 5%, $P < 0.01$, respectively). Up to 100 μM, the other prostanoids were not significantly affected. In conclusion, AF3442 is a selective mPGES-1 inhibitor which reduced monocyte PGE_2 generation also in the presence of plasma proteins. Pharmacological inhibition of mPGES-1 did not translate into redirection of PGH_2 metabolism towards other terminal PG synthases in monocytes. The functional relevance of this observation deserves to be investigated in vivo.

1. Introduction

Prostanoids are biologically active derivatives of arachidonic acid (AA) released from membrane phospholipids by phospholipases [1]. AA is transformed into prostaglandin (PG)H_2, through the activity of cyclooxygenase (COX) enzymes (i.e. COX-1 and COX-2), and subsequently PGH_2 is metabolized by terminal synthases to the biologically active prostanoids, i.e. prostacyclin (PGI_2), PGD_2, $PGF_{2\alpha}$, PGE_2 and thromboxane (TX)A_2 [1]. In particular, the isomerization of PGH_2 to PGE_2 is catalyzed by three different isomerases: a cytosolic PGE synthase (cPGES) and two membrane-bound PGESs, mPGES-1 and mPGES-2 [2]. Of these isomerases, cPGES and mPGES-2 are constitutive enzymes whereas mPGES-1 is mainly an induced isoform.

Several lines of evidence suggest that the mechanism of action of traditional non-steroidal anti-inflammatory drugs (tNSAIDs) and NSAIDs selective for COX-2 (named coxibs) for the treatment

Abbreviations: mPGES-1, microsomal prostaglandin (PG) E synthase-1; LPS, *Escherichia coli* lipopolysaccharide; TX, thromboxane; COX, cyclooxygenase; AF3442, [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-(trifluoromethyl)benzamide]; AA, arachidonic acid; PGI_2, prostacyclin; cPGES, cytosolic PGE synthase; tNSAIDs, traditional non-steroidal anti-inflammatory drugs; GI, gastrointestinal; MAPEG, membrane-associated proteins involved in eicosanoid and glutathione metabolism; TXS, TX synthase; PMSF, phenylmethylsulfonyl fluoride; FCS, fetal calf serum; TBS, Tris-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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of pain and inflammatory joint disease, is mainly through the inhibition of COX-2-dependent PGE_2 [3]. Inhibition of constitutively expressed COX-1 in gastrointestinal (GI) tract and presumably in platelets by tNSAIDs seems to play a role in increased risk of upper GI bleeding/perforation [4]. In fact, coxibs which spare COX-1 are associated with lower GI toxicity [3]. Although the inhibition of COX-2-dependent PGE_2 generation by tNSAIDs and coxibs is effective in ameliorating symptoms of inflammation and pain, a small but consistent increased risk of myocardial infarction has been detected in NSAID users [5–7]. Such adverse reactions are probably linked to the inhibition of COX-2-derived PGI_2 in vascular endothelial cells in the absence of an almost complete and persistent inhibition of platelet COX-1-derived TXA_2 [5–7]. Thus, a great interest is in the development of new anti-inflammatory drugs with a safer profile. Inhibitors of mPGES-1 are being developed for relief of pain – similarly to NSAIDs – but with the potential advantage of not affecting or perhaps causing a beneficial impact on the cardiovascular system [8,9]. Inhibitors of mPGES-1 might circumvent adverse GI side-effects of unselective COX inhibitors. However, in the stomachs of mice lacking mPGES-1 a shift from PGE_2 to all other prostanoids, i.e. TXA_2, PGD_2, $PGF_{2\alpha}$, and PGI_2, was detected thus making extremely difficult to predict the clinical consequences of mPGES-1 inhibition in the GI tract [10]. Further studies addressing this issue in appropriate animal models are required.

mPGES-1 belongs to the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily [11]. Similar to COX-2, mPGES-1 is highly up-regulated by proinflammatory stimuli and participates in elevated PGE_2 generation in inflammation [11]. It is functionally coupled with COX-2 [12], but differences in the promoter region between mPGES-1 and COX-2 [13] suggest divergent transcriptional mechanisms responsible for the inducible regulation of COX-2 and mPGES-1.

Deletion of mPGES-1 is associated with a profound inhibition of PGE_2 in vitro and in vivo and with a redirection of the PGH_2 substrate to other PG synthases, such as PGI_2 and PGD_2, which might participate to the safe cardiovascular profile [8,9]. Discrepant results have been found on the possible shift of PGH_2 metabolism to TXA_2 [8,14]. Whether redirection of PGH_2 metabolism towards other PG synthases might be influenced by the extent of reduction of mPGES-1 and by cell-specific features is unknown.

Development of biomarkers to predict drug responses is useful for a rational selection of doses for efficacy and toxicity. Endogenous mediators of inflammation might be used as biomarkers for therapeutic effects [15,16]. Thus, the assessment of COX-2 inhibition by the evaluation of PGE_2 levels in whole blood in response to *Escherichia coli* lipopolysaccharide (LPS) (an index of inducible COX-2 activity in monocytes) both in vitro and ex vivo (i.e. after dosing) [15,17] is considered a promising biomarker of drug response (analgesia, anti-inflammatory effects) in humans [15,16]. In fact, it has been shown that inhibition of LPS-induced PGE_2 by approximately 80% is sufficient to translate into clinical efficacy (analgesia) in humans [15].

The design, synthesis and characterization of a series of carbazole benzamides led us to the identification of several mPGES-1 inhibitors, among which AF3442 [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-(trifluoromethyl)benzamide] was identified as a lead compound and selected for further characterization [18].

In the present study, we aimed to characterize the contribution of mPGES-1 to PGH_2 metabolism in LPS-stimulated isolated human monocytes and whole blood by studying the synthesis of prostanoids and expression of COX-isozymes and down-stream synthases in the presence of pharmacological inhibition by AF3442. We choose to study the effect of the compound in human monocytes because they represent an important cell-target of the clinical efficacy of mPGES-1 inhibitors. Interestingly, we found that inhibition of PGE_2 generation at clinically relevant ranges was not associated with any significant change of other prostanoids in human monocytes in vitro. The functional relevance of this observation deserves to be investigated in vivo.

2. Materials and methods

2.1. Materials

AF3442 (Fig. 1) was synthesized and characterized in the Angelini Research Center [18]. MK-886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid) was purchased from Calbiochem (San Diego, CA, USA). RPMI-1640 and fetal calf serum (FCS) were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA). Interleukin-1β (IL-1β), penicillin/streptomycin, L-glutamine, LPS (derived from *E. coli* 026:B6), anti-rabbit peroxidase-conjugated IgG were from Sigma–Aldrich (St. Louis, MO, USA). PGH_2 substrate, COX-1, mPGES-1, mPGES-2, cPGES, TXS, PGIS polyclonal antibodies were from Cayman Chemical (Ann Arbor, MI, USA); β-actin and anti-goat peroxidase-conjugated IgG were purchased from Santa Cruz (USA). COX-2 polyclonal antibody was kindly provided by Dr. Stacia Kargman from Merk Frosst (Canada). Nucleofector Kit T was from Lonza (Cologne, AG, Germany). Ficoll-Paque and ECL plus were from GE Healthcare Life Sciences (Bucks, UK). Polyvinylidene fluoride (PVDF) membrane was purchased from Bio-Rad Laboratories (Hercules, CA).

2.2. Cloning and expression of human mPGES-1 in A549 cells

Cloning of human mPGES-1 was performed isolating mRNA from A549 (human lung adenocarcinoma) cells cultured in RPMI-1640 supplemented with 2% (v/v) FCS and stimulated with IL-1β (10 ng/ml) for 24 h. It was reverse transcribed into cDNA. Polymerase chain reaction amplification was then performed with specific primers [5′-CGGATCCGCCACCATGCCTGCCCACAGCCTG-3′ (FW) and 5′-GCTCTAGAGTCACAGGTGGCGGGCCGC-3′ (REV) containing BamHI and XbaI restriction sites, respectively] and fragments of the expected 459 bp size were obtained, ligated in the bacterial expression vector pcDNA3.1 and transformed into JM109 competent cells. Plasmids were isolated and miniprep products from a few colonies were subjected to restriction analysis that confirmed the presence of the expected fragment pattern. Two clones were sequenced in comparison with a sequence from EMBL Databank (accession number AF027740) and a conservative mutation in position 183 was found (a C replacing a T); however, the protein sequence alignement of the two clones with that of the reference mPGES-1 submitted in GenBank resulted in a 100% aminoacid identity. A549 cell lines were transfected in order to obtain a stable cell line overexpressing mPGES-1 enzyme. Transfection was performed in cuvettes by Nucleofector II

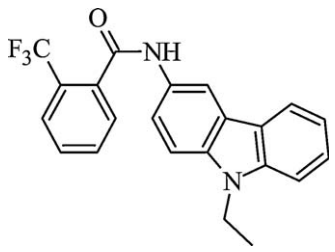

Fig. 1. Structure of AF3442 [*N*-(9-ethyl-9*H*-carbazol-3-yl)-2-(trifluoromethyl) benzamide].

(AMAXA) instrument using Nucleofector Kit T according to the manufacturer's instructions. The recombinant vector pcDNA3.1 (Neomicine resistant) ligated with human mPGES-1 was used for transfection. Transfection efficiency was assessed transfecting cells with a GFP (green fluorescent protein)-expressing vector and checking cell fluorescence by fluorescence microscopy after 4 and 24 h from transfection. After 48 h the medium was replaced with medium containing 400 μg/ml G418 (neomycin) as selecting agent. Cells were grown until neomycin selection in untransfected cells was confirmed. Clones were amplified and subsequently tested for mPGES-1 expression by Western blot [19] and for PGE_2 production by a validated radioimmunoassay [17]. Clone D8L was selected for its mPGES-1 high expression level.

2.3. Preparation of human mPGES-1-containing microsomal fraction

A549 and A549/human mPGES-1 (A549 clone D8L) cells maintained in culture were centrifuged, washed once with PBS and suspended at 10×10^6 cells/ml in lysis buffer [Tris–HCl 15 mM; sucrose 0.25 M; EDTA 0.1 mM; phenylmethylsulfonyl fluoride (PMSF) 2 mM; aprotinin 10 μg/ml; leupeptin 25 μg/ml; glutathione 1 mM]. The samples were kept on ice for 10 min. Then, the cells were lysed by 4× 30 s sonication pulses in a BANDELIN Soniplus sonifier at 70% of maximum power, frequency 9. Cell debris was removed by centrifugation at 10,000 × *g* for 15 min at 4 °C. The supernatant was centrifuged at 250,000 × *g* for 1 h and the membrane pellets were finally suspended in 0.1 ml of PGEG plus buffer (i.e. potassium phosphate 10 mM pH 7, glycerol 20%, EDTA 0.1 mM, glutathione 1 mM) containing PMSF 2 mM, aprotinin 10 μg/ml, leupeptin 25 μg/ml, for each milliliter of lysis buffer. Total protein amount was determined by the Bradford method (Bio-Rad Protein Assay) according to the manifacturer's instructions. Microsomal preparations were stored at −80 °C until used. The presence of the mPGES-1 protein in the microsomal preparation was confirmed by Western blot technique (Fig. 3A).

2.4. Inhibition of recombinant human mPGES-1

Aliquots of microsomal preparation from mPGES-1-overexpressing A549 clone D8L (30 μg/ml protein concentration) were used for assessing the enzymatic activity using MultiPROBE II Automated Liquid Handling System (PerkinElmer, Waltham, MA, USA) in the presence of 2.5 mM reduced glutathione and 10 μM PGH_2 substrate.

AF3442 (0.01–10 μM) was pre-incubated with reaction mixture containing microsomal preparation for 15 min at 4 °C. Reaction was started by substrate addition (PGH_2) and stopped after 5 min at 4 °C with 100 μl of stop solution (40 mM ferric chloride and 0.4 mM citric acid). Samples were then subjected to PGE_2 quantification by previously described radioimmunoassay technique [17]. MK-886 (0.1–100 μM), an inhibitor of MAPEG proteins, such as leukotriene C_4 synthase and 5-lipoxygenase activating protein (FLAP), affecting mPGES-1 activity at higher concentrations [20,21] was assessed, as positive control.

2.5. Isolation of human monocytes and pharmacological treatment

Human monocytes were separated from buffy coat by Ficoll-Paque, as previously described [17]. The contribution of COX-1 to prostanoid biosynthesis was minimized by pretreating the buffy coat with aspirin (300 μM). Mononuclear cell layer (obtained by Ficoll-Hypaque density gradient centrifugation) was treated with EDTA (2 mM) for 15 min at 37 °C to remove platelets specifically adherent to the monocytes [22]. Cell suspensions routinely contained >90% monocytes [17]. Monocytes ($1.5–2 \times 10^6$ cells/ml) cultured in RPMI-1640 supplemented with 0.5% (v/v) of FCS, 1% (v/v) penicillin/streptomycin and L-glutamine (2 mM), were incubated with LPS (10 μg/ml) (derived from *E. coli* 026:B6) at 37 °C for 24 h in the presence of DMSO vehicle or increasing concentrations (0.01–100 μM) of the mPGES-1 inhibitor, AF3442. This compound was dissolved in DMSO and 2 μl of vehicle or stock solution was added to 1 ml of cell suspension. PGE_2, TXB_2 (the stable hydrolysis product of TXA_2), $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (the hydrolysis product of PGI_2) levels were measured in cell culture media by previously described and validated radioimmunoassay techniques [17,19,23], while COX-2, COX-1, mPGES-1, mPGES-2, cPGES, TXS and PGIS protein levels were evaluated in cell lysates by previously described Western blot techniques [17,19].

2.6. Isolation of human platelets

Platelet-rich plasma was prepared by centrifugation of citrated blood at 200 × *g* for 15 min and platelets were isolated from plasma as previously described [24,25] and resuspended in RPMI-1640 supplemented with 0.5% (v/v) FCS. Platelet suspensions (5×10^8/ml; containing <1 leukocyte per 10,000 platelets) were analyzed for the levels of COX-2, COX-1, mPGES-1, mPGES-2, cPGES and TXS in cell lysates by Western blot technique [17,19].

2.7. Effects of AF3442 on prostanoid generation in human whole blood

Whole blood was drawn from 13 healthy volunteers (age range: 23–35 years). The study was approved by the local Ethics Committee and informed consent was obtained from each subject. The inhibitory effect towards constitutive PGE_2 generation was assessed by evaluating PGE_2 levels generated in whole blood allowed to clot for 60 min at 37 °C. In clotting whole blood, PGE_2 is mainly produced by platelets in response to endogenously generated thrombin through the activity of COX-1 [23–26] and cPGES and/or mPGES-2. In fact, mPGES-1 is not detectable in platelets (Fig. 2). The parallel measurement of TXB_2 (the major product of endogenous AA metabolism in platelets), $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ was performed to verify a possible off-target effect of AF3442 on COX-1. The effects of AF3442 on inducible PGE_2 generation were studied by assessing the levels of PGE_2 produced in heparinized human whole blood stimulated for 24 h with LPS (10 μg/ml), a stimulus for the induction of both COX-2 and mPGES-1 in monocytes [17,27]. The simultaneous measurements of TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were performed to verify the impact of AF3442 on COX-2 and the possible redirection of the accumulated PGH_2 substrate towards other PG synthases. For the assessment of the effects on constitutive PGE_2 generation, peripheral venous blood samples were drawn from healthy volunteers when they had not taken any NSAID during the 2 weeks preceding the study. For the assessment of the effects on LPS-induced PGE_2 generation, heparinized peripheral venous blood samples were drawn from the same subjects pretreated with 300 mg aspirin 48 h before sampling to suppress the contribution of platelet COX-1 activity. Different concentrations (0.001–100 μM) of AF3442 or DMSO vehicle (2 μl) were incubated with whole blood samples and then allowed to clot at 37 °C for 1 h or with heparinized whole blood samples in the presence of LPS (10 μg/ml) at 37 °C for 24 h. Prostanoids were measured in serum or plasma by previously described and validated radioimmunoassays [17,19,23]. In these assays the least detectable concentration was 1–2 pg/ml for all prostanoids.

2.8. Western blot analysis

Microsomal fractions or cell lysates samples were loaded (30 or 25–50 μg/lane, respectively) onto 4–15% SDS-PAGE and transferred to PVDF membrane. Membranes were saturated with a

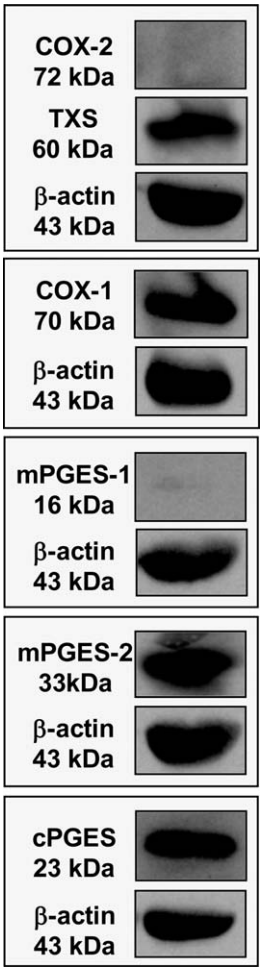

Fig. 2. Expression of COX-isozymes and down-stream synthases in isolated human platelets. Platelet-rich plasma was prepared by centrifugation of citrated blood at 200 × *g* for 15 min and platelets were isolated from plasma as previously described [24,25] and resuspended in RPMI-1640 supplemented with 0.5% (v/v) FCS (5×10^8 platelets/ml; containing <1 leukocyte per 10,000 platelets). Platelets were lysed and 25 μg of total proteins were analyzed for COX-1, COX-2, TXS, cPGES, mPGES-2, mPGES-1 and β-actin levels by SDS-PAGE and Western blot analysis [19]. The blot used for detection of COX-2 was stripped (stripping solution was from Chemicon International, USA) and reprobed for TXS. β-actin was used as protein loading control. The figure is representative of three different experiments.

solution of 5% non-fat milk in Tris-buffered saline–0.1% Tween-20 (TBS–Tween-20), and then incubated with anti-COX-2, anti-COX-1, anti-mPGES-1, anti-mPGES-2, anti-cPGES, anti-TXS, anti-PGIS, or anti-β-actin polyclonal antibodies for 1 h at room temperature [19]. Then, the membranes were washed in TBS–Tween-20 and incubated with the secondary antibodies: anti-goat peroxidase-conjugated IgG for β-actin or anti-rabbit peroxidase-conjugated IgG for the other proteins. Finally, the membranes were washed in TBS–Tween-20 and then all blots were developed by using ECL plus detection according to the manufacturer's instructions.

2.9. Statistical analysis

Results are expressed as mean ± SEM. For the experiments of LPS-stimulated monocytes and whole blood, the production of PGE_2, TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ was subtracted from the levels of the prostanoids measured in the presence of vehicle (DMSO). Statistical analysis was performed with Student's *t*-test or one-way ANOVA and Newman–Keuls multiple comparison test, using GraphPad Instat (version 3.00 for Windows). Values of $P < 0.05$ were considered statistically significant. Concentration–response curves were fitted

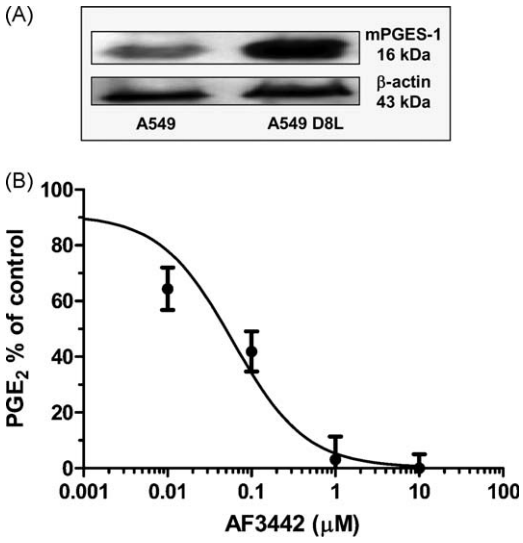

Fig. 3. Effect of AF3442 on microsomal preparations of A549 (human lung adenocarcinoma) cell line overexpressing human mPGES-1. (A) Western blot analysis of mPGES-1 in A549 (human lung adenocarcinoma) cell line and in those transfected with the recombinant vector pcDNA3.1 (Neomicine resistant) ligated with human mPGES-1 (clone D8L); (B) inhibitory effect of AF3442 on PGE_2 generation in microsomal preparations of A549 clone D8L. AF3442 (0.01–10 μM) was pre-incubated with reaction mixture containing microsomal preparations (30 μg/ml protein concentration) for 15 min at 4 °C, then the reaction was started by substrate addition (PGH_2) and stopped after 5 min at 4 °C with 100 μl of stop solution (40 mM ferric chloride and 0.4 mM citric acid). Samples were then subjected to PGE_2 quantification by radioimmunoassay. Results were reported as percentage of control (DMSO vehicle) (mean ± SEM) from three to four separate experiments.

(using PRISM, GraphPad, version 5.00 for Windows, San Diego, CA) and IC_{50} values were reported.

3. Results

3.1. Effect of AF3442 on human recombinant mPGES-1

As shown in Fig. 3B, AF3442 caused a concentration-dependent inhibition of PGE_2 generation by human recombinant mPGES-1 with and IC_{50} of 0.06 μM. In the same microsomal preparation, MK-886, used as a positive control, inhibited PGE_2 generation with an IC_{50} value of 4.1 μM (not shown), a comparable value to that found by Mancini et al. [20] (IC_{50}: 3.2 μM).

3.2. Expression of COX-isozymes and down-stream synthases and prostanoid generation in LPS-stimulated human monocytes

As shown in Fig. 4, in isolated human monocytes stimulated with LPS, COX-2 and mPGES-1 were profoundly induced. A slight, but reproducible, increase of cPGES by only 2-fold was detected. In contrast, we detected a constitutive expression of COX-1, TXS, mPGES-2 which was not affected by LPS. PGIS was barely detectable (Fig. 4). The spectrum of prostanoid generation (TXB_2, PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$) in monocytes both unstimulated and LPS-stimulated is reported in Table 1. LPS caused a significant increase of their generation. TXB_2 was the major product of AA (increased by 370-folds) while 6-keto-$PGF_{1\alpha}$ was the minor one (increased by 10-folds). PGE_2 and $PGF_{2\alpha}$ were increased at a similar extent (120-folds) (Table 1).

3.3. Effects of AF3442 on prostanoid generation in LPS-stimulated human monocytes

Incubation of LPS-stimulated monocytes for 24 h with AF3442 caused a concentration-dependent reduction of PGE_2 biosynthesis

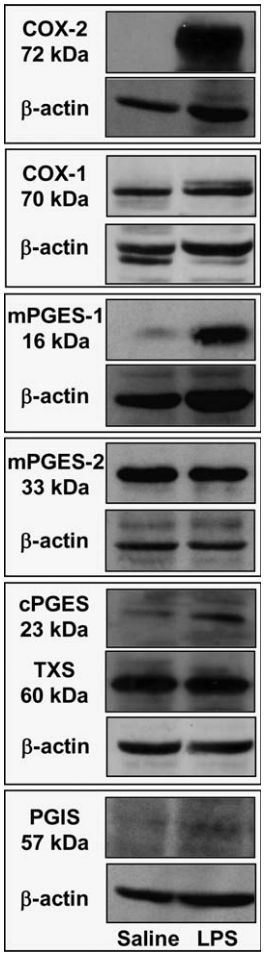

Fig. 4. Expression of COX-isozymes and down-stream synthases in LPS-stimulated human monocytes. Human monocytes were separated from buffy coat and 1.5–2 × 10^6 cells/ml were cultured in RPMI-1640 supplemented with 0.5% (v/v) FCS and incubated without (saline) or with LPS (10 μg/ml) at 37 °C for 24 h. Then, monocytes were lysed and 50 μg of total proteins were loaded onto 4–15% SDS-PAGE and analyzed by Western blot technique for COX-2, COX-1, TXS, cPGES, mPGES-1, mPGES-2, PGIS and β-actin expression. β-actin was used as protein loading control. The figure is representative of three different experiments.

with an IC_{50} value of 0.41 μM (Fig. 5). At 1 μM, AF3442 caused an inhibitory effect of 61 ± 3.3% ($P < 0.01$ versus DMSO vehicle) without significantly affecting the other prostanoids (TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$: 25.1 ± 5.1%, 21.1 ± 4.1%, 14.8 ± 4.8% of inhibition, respectively) (Fig. 5). Similar effects were found at 10 μM. In contrast, at 100 μM, we detected a higher reduction in the biosynthesis of PGE_2 (71.2 ± 2.4%, $P < 0.05$ versus 1 and 10 μM) (Fig. 5). This additional inhibitory effect of PGE_2 was associated with a significant reduction of TXB_2 generation of 54.7 ± 5.5% ($P < 0.01$ versus DMSO vehicle). A

Table 1
Prostanoid levels in LPS-stimulated human monocytes and whole blood.

Prostanoid	Monocytes (ng/10^6 cells)		Whole blood (ng/ml)	
	Saline	LPS	Saline	LPS
TXB_2	0.080 ± 0.011	29.7 ± 4.90*	0.890 ± 0.240	27.08 ± 3.66**
PGE_2	0.030 ± 0.005	3.70 ± 0.59*	0.840 ± 0.190	38.37 ± 8.03*,§
$PGF_{2\alpha}$	0.022 ± 0.004	2.69 ± 0.61*	0.500 ± 0.050	5.83 ± 0.64**
6-Keto-$PGF_{1\alpha}$	0.025 ± 0.010	0.30 ± 0.03**	0.005 ± 0.003	0.39 ± 0.09**

Values are reported as mean ± SEM ($n = 3$ for monocytes and $n = 9$–13 for whole blood).
* $P < 0.05$ versus saline.
** $P < 0.01$ versus saline.
§ $P < 0.05$ versus TXB_2.

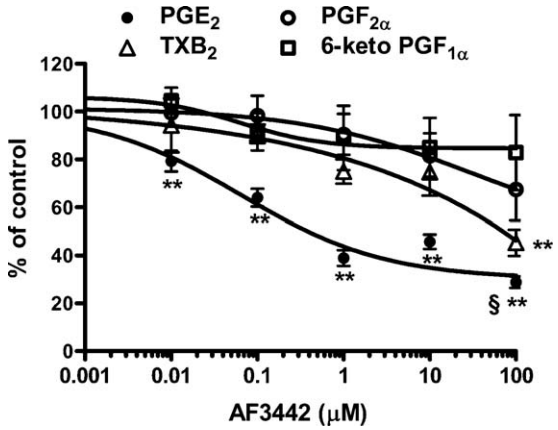

Fig. 5. Effects of AF3442 on prostanoid generation in LPS-stimulated human monocytes. Monocytes were separated from buffy coat which was pretreated with aspirin 300 μM for 5 min to suppress the contribution of COX-1 to prostanoid generation. Increasing concentrations of AF3442 (0.01–100 μM) or DMSO vehicle were incubated with human monocytes (1.5–2 × 10^6 cells/ml) in the presence of LPS (10 μg/ml) for 24 h at 37 °C. PGE_2, TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ levels were assayed in culture media by specific radioimmunoassays [17,19,23]. Results were reported as percentage of control (DMSO vehicle) (mean ± SEM) from three separate experiments. $^{\S}P < 0.05$, PGE_2 1 μM and 10 μM versus 100 μM and $^{**}P < 0.01$, PGE_2 0.01–100 μM and TXB_2 100 μM versus control.

lower reduction, but not significant, was detected for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (38.0 ± 8.9% and 29.0 ± 8.8%, respectively) (Fig. 5).

These data suggest a selective, but incomplete, inhibitory effect of AF3442 on mPGES-1 activity up to 10 μM. At 100 μM, PGE_2 reduction was associated with an inhibitory effect on TXB_2 suggesting a possible off-target effect on COX-2 activity and/or expression.

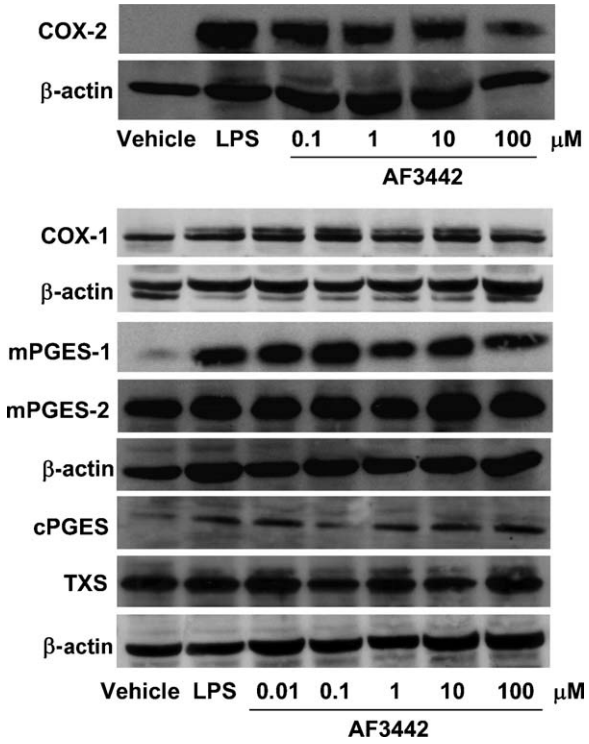

Fig. 6. Effects of AF3442 on the levels of COX-isozymes and down-stream synthases in LPS-stimulated human monocytes. Monocytes (1.5–2 × 10^6 cells/ml) were incubated with LPS (10 μg/ml) at 37 °C for 24 h in the presence of increasing concentrations (0.01–100 μM) of the mPGES-1 inhibitor, AF3442 or DMSO vehicle. COX-2, COX-1, mPGES-1, mPGES-2, cPGES, TXS and β-actin expression levels were evaluated in monocyte lysates by Western blot techniques. β-actin was used as protein loading control. The figure is representative of three different experiments.

3.4. Effects of AF3442 on the expression of COX-isozymes and down-stream synthases in LPS-stimulated human monocytes

As shown in Fig. 6, AF3442 up to 10 μM did not significantly affect the levels of COX-2, COX-1, mPGES-1, mPGES-2, cPGES and TXS. In contrast, at 100 μM, the compound caused a significant ($P < 0.01$ versus control) reduction only of COX-2 levels by 31.4 ± 2.6% (assessed as ratio of their optical density normalized to the optical density of β-actin).

3.5. Effects of AF3442 on prostanoid generation in human whole blood assays

To study whether the compound affected mPGES-1 activity even in the presence of plasma proteins, we assessed the effect of AF3442 on prostanoid generation in LPS-stimulated whole blood. Incubation of heparinized whole blood with LPS (10 μg/ml) resulted in a significant ($P < 0.01$) enhancement of PGE_2, TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production (Table 1). In this experimental model, PGE_2 and TXB_2 were the major prostanoids generated with PGE_2 that was higher than TXB_2 ($P < 0.05$) (Table 1). As reported in Fig. 7A, AF3442 inhibited in a concentration-dependent fashion LPS-induced PGE_2 biosynthesis with an IC_{50} of 29 μM. This value was 70-fold higher than that found in isolated human monocytes (in the presence of low plasma protein level: 0.5% FCS) suggesting an important binding of the compound to plasma proteins. A significant and selective inhibition of mPGES-1 activity was detected at 10 and 100 μM (38 ± 14%, $P < 0.05$ and 69 ± 5%, $P < 0.01$ versus vehicle, respectively). Up to 100 μM, the other prostanoids were not significantly affected (Fig. 7A).

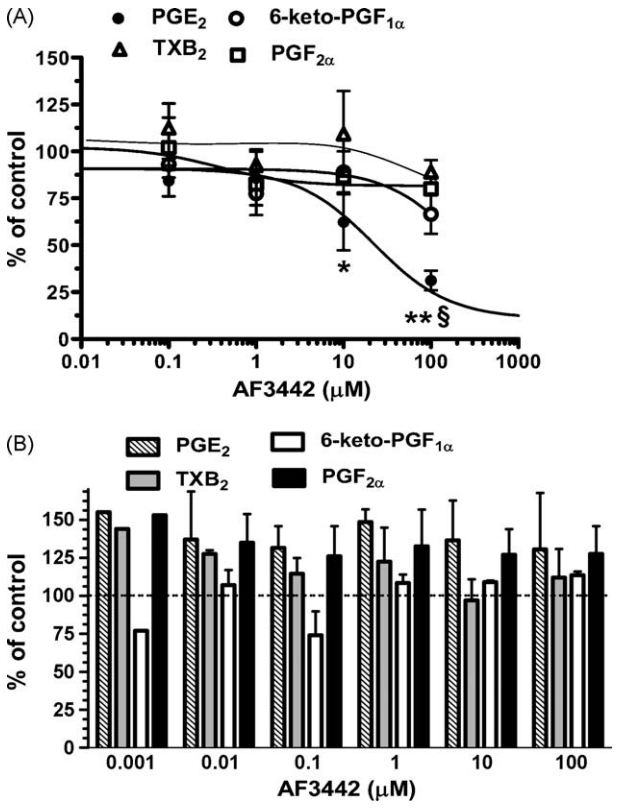

Fig. 7. Effects of AF3442 on LPS- and thrombin-induced prostanoids in human whole blood. (A) Effects of AF3442 on prostanid generation by whole blood COX-2-dependent pathway. Increasing concentrations of AF3442 (0.01–100 μM) or DMSO vehicle were incubated with 1 ml of heparinized human whole blood (drawn from healthy volunteers pretreated with 300 mg aspirin 48 h before sampling to reduce the contribution of platelet COX-1 to prostanoid generation) stimulated with LPS (10 μg/ml) for 24 h. Parallel assessment of PGE_2, TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (by radioimmunoassays) was performed to verify the selective inhibitory effect of the compound towards mPGES-1 and the possible redirection of PGH_2 metabolism to other PG synthases. Results were reported as % of control (DMSO vehicle) (mean ± SEM) from three separate experiments. $^{*}P < 0.05$, PGE_2 (% of control) at AF3442 10 μM versus vehicle (DMSO); $^{**}P < 0.01$, PGE_2 (% of control) at AF3442 100 μM versus vehicle (DMSO); $^{\S}P < 0.01$, PGE_2 versus $PGF_{2\alpha}$, TXB_2 and 6-keto-$PGF_{1\alpha}$ (% of control), at AF3442 100 μM. (B) Effects of AF3442 on prostanoid generation by whole blood COX-1-dependent pathway. AF3442 (0.001–100 μM) or DMSO vehicle was incubated with 1 ml of whole blood samples (drawn from healthy volunteers who did not take any NSAID for 2 weeks before blood collection) that were allowed to clot for 60 min to assess in vitro selective inhibiting effect of AF3442 towards COX-1-dependent prostanoids mainly of platelet origin [23,26]. Since platelets do not express mPGES-1, PGE_2 generation may occur by the activity of cPGES and/or mPGES-2. Thus, this assay allowed to verify the possible effects of the compound towards other PGE synthases. PGE_2, TXB_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ levels were measured by specific radioimmunoassays. Results were reported as % of control (DMSO vehicle) (mean ± SEM) from three separate experiments for AF3442 0.01–100 μM and from two experiments for AF3442 0.001 μM.

Then, we assessed the impact of AF3442 on thrombin-stimulated prostanoids which are generated mainly from platelet COX-1 and constitutive down-stream synthases. In fact, in platelets inducible COX-2 and mPGES-1 were not detectable (Fig. 2). As shown in Fig. 7B, the compound did not affect thrombin-stimulated PGE_2 and also other prostanoids.

Altogether, these results showed that the compound up to 100 μM – causing a clinically relevant degree of inhibition of PGE_2 by approximately 70% – is a selective inhibitor of mPGES-1 in human whole blood.

4. Discussion

Inhibitors of mPGES-1 are potential, novel anti-inflammatory drugs with the possible advantage of not affecting or perhaps causing a beneficial impact on cardiovascular homeostasis [9]. However, redirection of the PGH_2 substrate to other PG synthases in the presence of mPGES-1 deletion has been shown both in vivo and in vitro [8,14]. This phenomenon might occur at different extents in different cell types, thus, translating into the generation of prostanoids with opposite effects for the cardiovascular system [9]. It is unknown whether this effect occurs also in the presence of an incomplete inhibition of mPGES-1, as it would take place after drug treatment. Thus, we studied the contribution of mPGES-1 to PGH_2 metabolism in LPS-stimulated isolated human monocytes and whole blood in the presence of pharmacological inhibition by the novel mPGES-1 inhibitor, AF3442 [18]. We aimed to explore this effect in human monocytes because: (i) they are the target for clinical efficacy; (ii) they play a key role in atherogenesis through the generation of PGE_2, a mediator of inflammation, and TXA_2, a potent proaggregatory and vasoconstrictor agent [28]. AF3442 affected LPS-induced mPGES-1 activity of human monocytes even in the presence of plasma proteins, i.e. in whole blood (Fig. 7A). In whole blood at 100 μM, it caused a clinically relevant degree of inhibition of PGE_2 by approximately 70% which was not associated with any significant change of the biosynthesis of other prostanoids.

AF3442 is a molecule belonging to the carbazole class of compounds [18]. It was very potent towards recombinant human mPGES-1 (IC_{50}: 0.06 μM) and at 1 μM, it completely suppressed the enzyme activity. In LPS-stimulated monocytes (in the presence of low concentrations of proteins, i.e. 0.5% FCS), AF3442 1 μM caused a maximal, selective inhibition of PGE_2 of 61%. An almost comparable selective degree of inhibition of PGE_2 generation (by 69%) was obtained at 100 μM of the compound in LPS-stimulated whole blood which suggests an extensive plasma protein binding by the compound. An important issue is whether this extent of inhibition of LPS-stimulated whole blood PGE_2 by AF3442 might be clinically relevant. We have previously looked at the degree of inhibition of LPS-stimulated whole blood PGE_2 by circulating peak concentrations of therapeutic doses of several NSAIDs [6]. Our

results show that low-dose ibuprofen (1200 mg/day) caused a maximal inhibition of 75% [6]. Moreover, it has been shown that therapeutic plasma concentrations of analgesic doses of NSAIDs correlated with the IC_{80} (drug concentration which inhibits enzyme activity by 80%) for inhibition of LPS-stimulated whole blood PGE_2 production in vitro [15]. Altogether these findings suggest the possible clinical efficacy of AF3442 which should be confirmed by in vivo studies.

Failure of AF3442 to completely suppress inducible PGE_2 generation could be explained by the contribution of other PGES, such as mPGES-2 which may couple to COX-2, to the synthesis of this prostanoid [29]. Alternatively, it is possible that some PGH_2 isomerized to PGE_2, non-enzymatically [30,31]. However, in murine macrophages, in the presence of deletion of mPGES-1, PGE_2 generation was reduced by more than 90% demonstrating that mPGES-1 activity is a key factor in LPS-induced PGE_2 production, in this cell type [8,14].

Unexpectedly, we showed that in the presence of an incomplete suppression of mPGES-1, shunting of COX-2-derived PGH_2 to other prostanoids did not occur. This finding was showed both in whole blood and in isolated monocytes.

In human monocytes, LPS induced the generation of TXB_2, PGE_2, $PGF_{2\alpha}$ and extremely low levels of PGI_2. TXB_2 was the dominant product of monocytes exceeding by 10-fold PGE_2.

High levels of expression of TXS, together with efficiency of coupling between TXS and COX-2 [32,33] might be the determinants which contribute to the dominant generation of inducible TXA_2 in isolated monocytes. In fact, it has been shown that TXS efficiently couples to COX-2 when small amounts of AA are gradually supplied over a long period, such as in response to LPS [32,33]. In this scenario, mPGES-1 is not the dominant pathway of PGH_2 metabolism; thus its inhibition translates into a limited accumulation of PGH_2 which explains our failure to detect redirection towards TXB_2 and other prostanoids. This is different from the results obtained in thioglycollate-elicited peritoneal macrophages stimulated in vitro with LPS [8,14] showing that PGE_2 is the major prostanoid and deletion of mPGES-1 caused redirection towards TXB_2 and PGI_2, but TXB_2 predominated. However, Boulet et al. [10] studied the shunting of PGH_2 to alternative prostanoid pathways in the presence of complete and incomplete loss of mPGES-1 gene and they did not detect a significant redirection towards TXB_2 in peritoneal macrophages under similar experimental conditions. The reason of these discrepant results is unclear. It has been suggested that the use of mice with different genetic backgrounds might have played a role [14].

We propose that differential expression of influx and efflux transport proteins may influence PGH_2 redirection towards other PG synthases. Excess PGH_2 may be immediately released outside the cell after intracellular biosynthesis – probably through the action of MRP4/ABCC4 (multidrug resistance protein 4 adenosine triphosphate-binding cassette, subfamily C, member 4) [34] or simple diffusion – and then it may be reuptaken, probably via the prostaglandin transporter [35,36]. Thus, shunting of PGH_2 to other prostanoids may be dependent on the expression of transport proteins in different cell types. This hypothesis will be explored in a specific study.

In order to verify the occurrence of PGH_2 redirection in the presence of mPGES-1 inhibition in a more physiologic system, we studied the effect of AF3442 in LPS-stimulated whole blood where TXB_2, PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ are mainly from monocytes through the activity of induced COX-2 [17]. In this system, PGE_2 is the major prostanoid generated which was only slightly more abundant than TXB_2 (Table 1). The lower concentration of PGE_2 generated in isolated monocytes versus whole blood may suggest the contribution of extramonocyte mPGES-1 to the generation of PGE_2 in whole blood. In particular, neutrophil mPGES-1, which has been shown to be constitutively expressed [37], might act a part. However, even in whole blood where PGE_2 was the major prostanoid, we did not detect significant redirection of PGH_2 metabolism towards other prostanoids. These results may suggest that accumulated PGH_2 is rapidly released and not reuptaken by blood cells. Released PGH_2 may bind to plasma proteins with avidity thus preventing transcellular metabolism of PGH_2 between blood cells and leading to its degradation.

Deletion of mPGES-1 retards atherogenesis and limits aortic aneurysm formation in hyperlipidaemic mice [8,9]. However, it does not predispose to thrombogenesis and has a limited impact on blood pressure compared to inhibition of COX-2 [8,9]. These favourable effects for the cardiovascular system, in the presence of deletion of mPGES-1, may at least in part be due to augmented excretion of major metabolites of PGD_2 (PGD-M) and PGI_2 (PGI-M) [8,9,38]. Enhanced synthesis of PGI_2 in mPGES-1 deleted murine macrophages and vascular smooth muscle cells in vitro was found by Wang et al. [8]. We did not confirm this phenomenon in human monocytes by the novel mPGES-1 inhibitor in vitro. However, it should be pointed out that in these cells PGI_2 was generated at very low concentrations which is consistent with the very low levels of expression of PGIS detected in monocytes (Fig. 4). The occurrence of increased biosynthesis of PGI_2 in vivo by pharmacological inhibition of mPGES-1 should be verified.

In human monocytes, in the absence of plasma proteins, a concentration of AF3442 100 μM, i.e. 100-fold higher than that causing a maximal inhibition of mPGES-1, was associated with a small off-target effect on COX-2 expression. Whether down-regulation of COX-2 levels by 35% involved a transcriptional and/or a posttranscriptional mechanism remains to be investigated.

In conclusion, we have demonstrated that a selective mPGES-1 inhibitor reduced monocyte PGE_2 generation at clinically relevant ranges (i.e. approximately 70%) also in presence of plasma proteins. In human monocytes, mPGES-1-dependent PGH_2 metabolism represents a major metabolic route of PGE_2 generation, while other PGESs or non-enzymatic formation of PGE_2 represent minor pathways of this prostanoid biosynthesis. Pharmacological inhibition of mPGES-1 did not translate into redirection of PGH_2 metabolism towards other terminal PG synthases in monocytes. The functional consequences of the phenotype induced by pharmacological inhibition of mPGES-1 deserve to be investigated in vivo in suited experimental animals.

Conflict of interest

Isabella Coletta, Giorgina Mangano, Maria Alessandra Alisi, Lorenzo Polenzani, Claudio Milanese are employees of A.C.R.A.F. S.p.A., Angelini Research Center who funded this research. Paola Patrignani received a grant by A.C.R.A.F. to perform the study.

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