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R(+)-METHANANDAMIDE AND OTHER CANNABINOIDS INDUCE THE EXPRESSION OF CYCLOOXYGENASE-2 AND MATRIX METALLOPROTEINASES IN HUMAN NON-PIGMENTED CILIARY EPITHELIAL CELLS [#]

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Running title: COX-2 and MMP expression by cannabinoids

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Abbreviations: AEA, anandamide (arachidonylethanolamide); AM-251, [N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]; AM-630, [(6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl) (4-methoxyphenyl)methanone]; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; COX, cyclooxygenase; IOP, intraocular pressure; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NPE, non-pigmented ciliary epithelium; NSAID, non-steroidal anti-inflammatory drug; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; PD98059, 2´-amino-3´-methoxyflavone; PG, prostaglandin; POAG, primary open-angle glaucoma; R(+)-MA, R(+)-methanandamide (R-(+)-arachidonyl-1'-hydroxy-2'-propylamide); RT-PCR, reverse transcriptase-polymerase chain reaction; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)imidazol; Δ^9-THC, Δ^9-tetrahydrocannabinol; TIMP,

tissue inhibitor of matrix metalloproteinase; TRPV1, transient receptor potential vanilloid 1 or vanilloid receptor 1; WST-1, (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene disulfonate).

ABSTRACT

Prostaglandins (PGs) and matrix metalloproteinases (MMP) have been implicated in lowering intraocular pressure (IOP) by facilitating aqueous humor outflow. A possible role of cyclooxygenase-2 (COX-2) in this process was emphasized by findings showing an impaired COX-2 expression in the non-pigmented ciliary epithelium (NPE) of patients with primary open-angle glaucoma. Using human NPE cells, the present study therefore investigated the effect of the IOP-lowering cannabinoid R(+)-methanandamide (R(+)-MA) on the expression of COX-2 and different MMPs and tissue inhibitors of MMPs (TIMPs). R(+)-MA led to a concentration- and time-dependent increase of COX-2 mRNA expression. R(+)-MA-induced COX-2 expression was accompanied by time-dependent phosphorylations of p38 mitogen-activated protein kinase (MAPK) and p42/44 MAPK, and was abrogated by inhibitors of both pathways. Moreover, R(+)-MA increased the mRNA and protein expression of MMP-1, -3, -9 and TIMP-1, but not that of MMP-2 and TIMP-2. Inhibition of COX-2 activity with NS-398 was associated with a virtually complete suppression of R(+)-MA-induced MMP-9 and TIMP-1 expression. Consistent with this data, MMP-9 and TIMP-1 expression was also induced by PGE_2, a major COX-2 product. Two other COX-2-inducing cannabinoids, anandamide and Δ^9-tetrahydrocannabinol, caused the same pattern of MMP- and TIMP expression as R(+)-MA both in the absence and presence of NS-398. Altogether, cannabinoids induce the production of several outflow-facilitating mediators in the human NPE. Our results further imply an involvement of COX-2-dependent PGs in MMP-9 and TIMP-1 expression. In conclusion, stimulation of intraocular COX-2 and MMP expression may represent a potential mechanism contributing to the IOP-lowering action of different cannabinoids.

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INTRODUCTION

Intraocular pressure (IOP) is regulated on the levels of aqueous humor formation and aqueous humor outflow via the trabecular meshwork and the uveoscleral route. In recent years, a coordinated turnover of the extracellular matrix by matrix metalloproteinases (MMPs) has been proposed to underly the outflow process. Several studies indicate that this homeostasis is out of balance in primary open-angle glaucoma (POAG) (La Rosa and Lee, 2000; Schlötzer-Schrehardt et al., 2003). Moreover, recent evidence suggests that induction of MMPs plays a role in numerous glaucoma therapies, including topical prostaglandin (PG) analogs (Weinreb and Lindsey, 2002) and argon laser trabeculoplasty (Parshley et al., 1996). For instance, increases in the biosynthesis of certain MMPs and subsequent remodeling of the extracellular matrix appear to be critical for latanoprost´s facilitating action on aqueous outflow through the ciliary muscle (Weinreb et al., 2002).

Likewise, endogenous PGs have been implicated in the reduction of IOP by facilitating the outflow of aqueous humor (Weinreb et al., 2002). Evidence suggesting a role of cyclooxygenase (COX)-2-derived PGs in this process comes from a recent study of our laboratory showing that COX-2 is constitutively expressed in the non-pigmented secretory epithelium (NPE) of the ciliary body, but is completely lost in the NPE of patients with end-stage POAG (Maihöfner et al., 2001). However, the precise function of COX-2 in the context of IOP regulation is poorly understood. As COX-2-dependent PGs have been implicated in the induction of MMPs in different cell types (Zhang et al., 1998; Callejas et al., 2001; Ottino and Bazan, 2001), it is tempting to speculate that COX-2 induced by antiglaucomatous substances may modulate the expression of these enzymes in tissues involved in IOP regulation. Precisely because the NPE is the primary source of aqueous humor, COX-2-derived PGE_2 and MMPs synthesized by these cells and secreted into aqueous humor

subsequently, could be involved in the degradation of extracellular matrix of both uveoscleral and trabecular tissues.

During past years cannabinoids have received considerable attention as potential antiglaucomatous drugs (Järvinen et al., 2002). The IOP-lowering action of Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal psychoactive component of marijuana, was first reported in 1971 (Hepler und Frank, 1971). In the following years, various human or animal studies have confirmed IOP decreases by marijuana, Δ^9-THC and classic cannabinoid derivatives (Flom et al., 1975; Purnell and Gregg, 1975; Colasanti, 1986). Likewise, the endogenous cannabinoid, anandamide (arachidonylethanolamide, AEA), and its stable analog, R(+)-methanandamide (R(+)-MA), have meanwhile been shown to cause IOP reduction (Pate et al., 1995; Juntunen et al., 2003). Moreover, in eyes from patients with glaucoma, significantly decreased levels of the endocannabinoid 2-arachidonoylglycerol and the AEA congener, palmitoylethanolamide, were detected in the ciliary body, suggesting that endocannabinoids may have a role in this disease, particularly with respect to regulation of IOP (Chen et al., 2005). Despite this, the mechanism underlying the IOP-lowering and antiglaucomatous action of cannabinoids remains to be established. On the basis of inhibitor studies with non-steroidal antiinflammatory drugs (NSAIDs), the IOP-lowering effects of AEA and Δ^9-THC have been associated with the generation of PGs (Pate et al., 1996; Green et al., 2001). However, no further mechanistic studies have been performed in this context so far. Interestingly, reduction of IOP by AEA has been proposed to occur in a CB_1 receptor-independent manner (Pate et al., 1998).

To investigate the mode of action of IOP-lowering cannabinoids with respect to the production of outflow-facilitating mediators, the present study addressed the effect of R(+)-MA and other cannabinoids on the generation of COX-2-dependent PGs and MMPs in human NPE cells. In addition, a possible association of COX-2 with the induction of MMPs

was analyzed. To this end we used the immortalized NPE cell line ODM-2, which has been established as a valuable model to study molecular pathways in the human NPE (Martin-Vasallo et al., 1989). Our data show that R(+)-MA as well as AEA and Δ^9-THC induce the generation of COX-2-dependent PGs, a broad spectrum of MMPs (MMP-1, -3, -9) and a tissue inhibitor of MMPs (TIMP-1). In case of cannabinoid-induced formation of MMP-9 and TIMP-1, de novo expressed COX-2 was shown to play a crucial role in this response. It is proposed that the induction of COX-2- and MMP expression in the NPE may represent a potential mechanism contributing to the IOP-lowering action of R(+)-MA and other cannabinoids.

MATERIAL AND METHODS

Materials.

AEA, AM-251, AM-630, capsazepine, NS-398 and PGE_2 were bought from Alexis Deutschland GmbH (Grünberg, Germany). SB203580, PD98059, pertussis toxin and R(+)-MA were purchased from Calbiochem (Bad Soden, Germany). Δ^9-THC was obtained from Sigma Chemie (Deisenhofen, Germany). Dulbecco´s modified Eagle´s medium with 4 mM L-glutamine and 4.5 g/l glucose was from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium). Fetal calf serum and penicillin-streptomycin were obtained from PAN Biotech (Aidenbach, Germany) and Invitrogen (Karlsruhe, Germany), respectively.

Cell culture.

SV40-transformed human NPE cells (ODM-2 cells) were kindly provided by Dr. M. Coca-Prados (New Haven, CT, USA). Cells were maintained in Dulbecco´s modified Eagle´s medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2. All incubations were performed in serum-free medium.

Quantitative RT-PCR analysis.

NPE cells (125,000 cells per well) were grown to confluence in 24-well plates. Following incubation of cells with the respective test compounds or its vehicles for the indicated times, supernatants were removed and cells were lysed for subsequent RNA isolation. Total RNA was isolated using the RNeasy total RNA Kit (Qiagen, Hilden, Germany). ß-Actin- (internal standard), COX-2-, MMP- and TIMP mRNA levels were determined by quantitative real-time RT-PCR. Briefly, this method uses the 5´→3´exonuclease activity of the Taq polymerase to

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cleave a probe during PCR. A probe consists of an oligonucleotide coupled with a reporter dye (6-carboxyfluorescein; 6FAM) at the 5´end of the probe and a quencher dye (6-carboxy-tetramethylrhodamine; TAMRA) at an internal thymidine. Following cleavage of the probe, reporter and quencher dye become separated, resulting in an increased fluorescence of the reporter. Accumulation of PCR products was detected directly by monitoring the increase in fluorescence of the reporter dye using the integrated thermocycler and fluorescence detector ABI PRISM™ 7700 Sequence Detector (Perkin Elmer, Weiterstadt, Germany). Quantification of mRNA was performed by determining the threshold cycle (C_T), which is defined as the cycle at which the 6FAM fluorescence exceeds 10 times the standard deviation of the mean baseline emission for cycles 3 to 10. COX-2-, MMP- and TIMP mRNA levels were normalized to ß-actin according to the following formula: C_T (gene of interest) – C_T (ß-actin) = ΔC_T. Subsequently, mRNA levels of the gene of interest were calculated using the $\Delta\Delta C_T$ method: ΔC_T (test compound) - ΔC_T (vehicle) = $\Delta\Delta C_T$ (test compound). The relative mRNA level for the respective test compound was calculated as $2^{-\Delta\Delta C_T} * 100\%$. RT-PCR reaction was performed using the One Step RT-PCR kit (Qiagen, Hilden, Germany). RNA samples were amplified using specific primers for human ß-actin and COX-2 (TIB MOLBIOL, Berlin, Germany) as described previously (Ramer et al., 2001). Primers and probes for human MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 were assays-on-demand™ gene expression products (Applied Biosystems, Darmstadt, Germany).

Western blot analysis.

Cells seeded in 10-cm dishes (1,250,000 cells per dish) and grown to confluence were incubated with the respective test compounds or its vehicles for the indicated times. Afterwards, NPE cells were washed, harvested, and pelleted by centrifugation. Cells were then lysed in solubilization buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton® X-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml

leupeptin and 10 µg/ml aprotinin), homogenized by sonication, and centrifuged at 10,000 x g for 5 min. Supernatants were used for Western blot analysis of the COX enzymes and the mitogen-activated protein kinases (MAPKs) p38 and p42/44.

For determination of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 protein, cell culture supernatants were centrifuged and concentrated 10-fold using Microcon YM-10 centrifugal filter units (Millipore GmbH, Schwalbach, Germany) with a 10 kDa cut-off. For this purpose 500-µl aliquots of supernatants were pipetted on filter units and centrifuged for 90 min at 14,000 x g and at 4 °C. The final volumes of the retentants were adjusted and total protein was measured using a bicinchoninic acid assay (Pierce, Rockford, IL, USA).

For all Western blot analyses 60 µg protein were loaded and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Following transfer to nitrocellulose and blocking of the membranes with 5% milk powder, blots were probed with specific antibodies raised to COX-2 (BD Biosciences, Heidelberg, Germany), COX-1 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), p38 MAPK, phospho-p38 MAPK, p42/44 MAPK, phospho-p42/44 MAPK (all from New England BioLabs GmbH, Frankfurt, Germany), MMP-1**,** MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 (all from Oncogene Research Products, San Diego, CA). Primary antibodies were allowed to react for 1 h at room temperature (COX-1, COX-2, p38 MAPK, phospho-p38 MAPK, p42/44 MAPK, phospho-p42/44 MAPK) or overnight at 4 °C (MMP-1**,** MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2). Subsequently, membranes were probed with horseradish peroxidase-conjugated Fab-specific anti-mouse IgG (Sigma, Deisenhofen, Germany) and horseradish peroxidase-linked anti-rabbit IgG (New England BioLabs GmbH, Frankfurt, Germany), respectively. Antibody binding was visualized by enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences Inc., Freiburg, Germany).

Determination of PGE_2.

Cells (125,000 per well) grown to confluence in 24-well plates were incubated with test substances or its vehicles for the indicated times. PGE_2 concentrations in cell culture supernatants were determined using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Cell viability assay.

Cells (40,000 per well) were seeded in 96-well flat bottom microplates and were grown to confluence. Subsequently, cells were incubated with vehicle or different concentrations of R(+)-MA for 48 h in 100 µl medium without serum. Afterwards, cell viability was measured by the colorimetric WST-1 test (Roche Diagnostics, Mannheim, Germany). This cell viability test is based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene disulfonate) by mitochondrial dehydrogenases in metabolically active cells.

Statistics.

All values are presented as means ± S.E.M. Comparisons between groups were performed with Student´s two-tailed t-test.

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RESULTS

Time-course and COX-2-dependency of R(+)-MA-induced PGE_2 synthesis.

Incubation of human NPE cells with R(+)-MA (10 µM) for up to 24 h led to a continuous increase of PGE_2 levels in cell culture supernatants during the first 8 h up to a 3.3-fold induction over vehicle (Fig. 1A). Significant induction of PGE_2 synthesis by R(+)-MA became evident at 2 h after stimulation. An approximately 2.8-fold induction of PGE_2 synthesis was observed even at a stimulation period as long as 24 h (Fig. 1A).

To examine the source of PGE_2 release, cells were treated with R(+)-MA in the presence of the selective COX-2 inhibitor NS-398. NS-398 completely abolished R(+)-MA-induced PGE_2 release, confirming an involvement of the COX-2 isozyme in this response (Fig. 1B). In accordance with this finding, celecoxib, another COX-2-selective inhibitor, led to a complete inhibition of R(+)-MA-induced PGE_2 synthesis at a concentration as low as 0.1 µM (data not shown).

As determined by the WST-1 test, R(+)-MA at 10 µM did not show a cytotoxic effect on human NPE cells over a 48-h incubation period (data not shown). Decreased viability of cells was first registered at an R(+)-MA concentration of 30 µM (67.1 ± 0.25%; n = 4; $P < 0.001$, vs. vehicle; Student´s *t*-test, relative to viability of vehicle-treated cells [100%]).

Time-course and concentration-dependency of R(+)-MA-induced COX-2 expression.

To determine whether induction of PGE_2 synthesis by R(+)-MA was reflected in the expression of COX-2, COX-2 mRNA levels were analyzed using real-time RT-PCR. Incubation of cells with R(+)-MA resulted in a sustained stimulation with COX-2 mRNA peak levels after 8- and 12-h incubation periods (Fig. 2A). A significant induction of COX-2 expression was still evident following a 24-h incubation with the cannabinoid (Fig. 2A).

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Further analyses revealed a concentration-dependent increase of COX-2 mRNA levels by R(+)-MA (Fig. 2B).

To determine whether induction of COX-2 mRNA by R(+)-MA was reflected in the expression of protein encoded by this mRNA, COX-2 protein levels were further analyzed. Western blot analysis of cell lysates from R(+)-MA-treated cells revealed an increase of COX-2 protein levels (Fig. 2C). Under the same experimental conditions the expression of COX-1 remained unchanged (Fig. 2C). The stimulatory effect of R(+)-MA on COX-2 protein expression was mimicked by the structurally related AEA as well as by the phytocannabinoid Δ^9-THC (Fig. 2D). Likewise, both compounds induced COX-2 expression at the transcriptional level. Accordingly, COX-2 mRNA levels in cells treated with AEA and Δ^9-THC at 10 µM each for 8 h were 244 ± 17% (n = 4; $P < 0.001$, vs. vehicle; Student´s *t*-test) and 319 ± 13% (n = 3; $P < 0.001$, vs. vehicle; Student´s *t*-test), respectively, relative to COX-2 mRNA levels in vehicle-treated cells (100%).

Time-course of R(+)-MA-induced p38 and p42/44 MAPK phosphorylation.

Since MAPK activation has been implicated in the expression of immediate-early genes, the involvement of these enzymes in R(+)-MA-induced COX-2 expression by NPE cells was investigated by Western blots using antibodies against the phosphorylated forms of p38 and p42/44 MAPKs. As internal standards blots were incubated with antibodies to the unphosphorylated forms of p38 and p42/44 MAPK. To examine the time-course of phosphorylation of both kinases by R(+)-MA, NPE cells were treated with the cannabinoid at 10 µM for up to 4 h. According to Fig. 3A, the activation of p38 MAPK occurred in a biphasic manner with increases in phosphorylation becoming evident after stimulation periods of 5 and 30 min, respectively. Second-burst phosphorylation was still evident at 240 min post-stimulation. Phosphorylation of p42/44 MAPK began to increase within 5 min after the

addition of R(+)-MA, reached maximal stimulation at 15 min that sustained for at least 60 min (Fig. 3B). As with p38, a significant phosphorylation was still evident at 240 min post-stimulation.

Characteristics of R(+)-MA-induced COX-2 expression.

To confirm a causal link between activation of p38 and p42/44 MAPKs and induction of COX-2 expression by R(+)-MA, the impact of specific inhibitors of p38 MAPK (SB203580) and p42/44 MAPK activation (PD98059) on COX-2 expression was assessed in further experiments. According to Fig. 4A, R(+)-MA-induced COX-2 mRNA expression was totally abolished in the presence of both inhibitors at concentrations of 10 µM. The same concentration of SB203580 and PD98059 had no influence on basal COX-2 expression (data not shown). To ascertain a possible role of cannabinoid receptors and transient receptor potential vanilloid 1 (TRPV1) in the stimulatory action of R(+)-MA on COX-2 expression, R(+)-MA-treated cells were preincubated with the CB_1 receptor antagonist AM-251, the CB_2 receptor antagonist AM-630 or the TRPV1 antagonist capsazepine. All inhibitors were used at a standard concentration of 1 µM which has been reported to be within the range of concentrations inhibiting CB_1-, CB_2- and TRPV1 receptor-dependent events (Jacobsson et al., 2001; Mukherjee et al., 2004). However, all three substances did not suppress R(+)-MA-induced COX-2 expression (Fig. 4B). In further experiments, a 24-h pretreatment of cells with the $G_{i/o}$ protein inactivator, pertussis toxin (100 ng/ml), left R(+)-MA-induced COX-2 expression virtually unaltered (data not shown).

Time-course of R(+)-MA-induced mRNA expression of different MMPs and TIMPs.

To study potential functional consequences of R(+)-MA-induced COX-2 expression, we next analyzed mRNA levels of different MMPs and TIMPs in R(+)-MA-stimulated NPE cells. In

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NPE cells R(+)-MA produced significant increases of MMP-1, MMP-3, MMP-9 and TIMP-1 mRNA levels. Respective effects were observed following incubation periods as long as 12 h (MMP-3) or 24 h (MMP-1, -9, TIMP-1) (Fig. 5). In contrast, mRNA levels of MMP-2 and TIMP-2 did not show significant alteration (Fig. 5).

Effect of NS-398 on R(+)-MA-induced MMP and TIMP expression.

In an attempt to link products of COX-2 induction to the observed increases in the expression of MMP and TIMP expression, cells were treated with R(+)-MA in the presence of NS-398 at a concentration that totally abolished R(+)-MA-induced PGE_2 synthesis. As shown in Fig. 6, NS-398 almost completely suppressed R(+)-MA-elicited mRNA expression of MMP-9 and TIMP-1 by 91% and 81%, respectively. In case of MMP-1 and MMP-3, NS-398 diminished R(+)-MA-induced mRNA increases by 36% and 35%, respectively (Fig. 6). However, inhibitions did not reach statistical significance.

Corresponding with the mRNA data, a similar profile was observed at the protein level. Again, NS-398 profoundly suppressed R(+)-MA-induced MMP-9 and TIMP-1 levels (Fig. 7), whereas no substantial change was observed in case of R(+)-MA-induced MMP-1 and MMP-3 (Fig. 7). In line with the RT-PCR data, R(+)-MA did not induce MMP-2 and TIMP-2 protein levels in NPE cells (Fig. 7).

Modulation of the expression of MMPs and TIMPs by PGE_2.

To further confirm an involvement of COX-2 in MMP-9- and TIMP-1 induction, Western blot analyses were performed using the major COX-2 product PGE_2. According to Fig. 8, PGE_2 induced the expression of MMP-9 and TIMP-1. By contrast, protein levels of MMP-2, MMP-3 and TIMP-2 were not enhanced by PGE_2 (Fig. 8). An induction of MMP-1 expression by PGE_2 was already shown in a recent study from this laboratory (Hinz et al.,

2005). In line with the protein data, PGE_2 elicited a time-dependent induction of MMP-9 and TIMP-1 expression at the mRNA level (Fig. 9). Again, there was no significant alteration of MMP-2, MMP-3 and TIMP-2 mRNA expression over the 24-h incubation period studied (data not shown).

Regulation of MMP- and TIMP expression by AEA and Δ^9-THC.

To determine whether the effects on MMP- and TIMP synthesis were unique for R(+)-MA or shared by other COX-2 inducing cannabinoids, additional experiments were performed with the structurally related AEA and the phytocannabinoid Δ^9-THC. According to the results shown in Fig. 10 both compounds elicited virtually the same pattern of MMP- and TIMP expression as R(+)-MA. In further accordance with the R(+)-MA data, inhibition of PGE_2 synthesis by NS-398 caused a significant decrease in both AEA- and Δ^9-THC-induced MMP-9- and TIMP-1 expression (Fig. 10).

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DISCUSSION

Recent investigations suggest that cannabinoids mediate IOP reduction, at least in part, through the release of endogenous PGs (Pate et al., 1996; Green et al., 2001). However, the molecular mechanisms as well as the functional consequence of the putative PG-elevating action of these compounds is poorly understood**.**

The present study demonstrates a stimulatory effect of the endocannabinoid derivative, R(+)-MA, on the expression of the PG-synthesizing enzyme COX-2 in human NPE cells. Enhanced expression of COX-2 was accompanied by increased levels of PGE_2 in cell culture supernatants. The involvement of COX-2 in the PG-elevating effect of R(+)-MA was confirmed by experiments demonstrating that the selective inhibitor of COX-2 activity, NS-398, totally abolished this response. Interestingly, significant increases in PGE_2 synthesis became already evident at 2 h post-stimulation. Although we have not further addressed this issue, it is tempting to speculate that mechanisms involving liberation of arachidonic acid by phospholipase A_2 are involved in this early response. Indeed, AEA has previously been shown to activate the phospholipase A_2 enzyme (Wartmann et al., 1995).

To provide evidence for targets within the R(+)-MA-mediated COX-2 induction, our interest has focused on the MAPK signaling cascade that has been linked to COX-2 expression (Dean et al., 1999; Ramer et al., 2001, 2003). Western blot analysis using antibodies specific for phospho-p38 MAPK and phospho-p42/44 MAPK revealed an activation of both kinases by R(+)-MA. Consistent with these findings, R(+)-MA-induced COX-2 expression was markedly suppressed by SB203580, a selective p38 MAPK inhibitor, and PD98059, a specific inhibitor of p42/44 MAPK activation, confirming that both p38 and p42/44 MAPKs play a crucial role in mediating up-regulation of COX-2 by R(+)-MA.

Additional experiments using selective antagonists of CB_1- (AM-251) or CB_2 receptors (AM-

630) and the TRPV1 receptor antagonist capsazepine revealed that neither cannabinoid nor TRPV1 receptors confer R(+)-MA-induced COX-2 expression. Likewise, there was no alteration of COX-2 induction in the presence of the $G_{i/o}$ protein inactivator pertussis toxin, which further excludes an involvement of pertussis toxin-sensitive CB receptors as well as previously postulated pertussis toxin-sensitive non-CB_1/non-CB_2 G-protein-coupled cannabinoid receptor subtypes (Sagan et al., 1999) in this response. Collectively, these data support previous observations from our group showing a receptor-independent induction of COX-2 expression by R(+)-MA in human neuroglioma cells (Ramer et al., 2001, 2003). A possible mode of action of the lipophilic R(+)-MA may lie in alterations of membrane fluidity which in turn may lead to activation of second messengers conferring MAPK activation. In a recent study, cholesterol-rich membrane lipid rafts have been proposed to confer COX-2 expression by R(+)-MA (Hinz et al., 2004). With respect to the receptors involved in the IOP-lowering action of cannabinoids, different results have been published. Whereas the synthetic CB_1 agonist WIN55212-2 has been shown to reduce IOP in rabbits CB_1 receptor-dependently (Song and Slowey, 2000), AEA has been demonstrated to elicit a respective response in the same species via a CB_1 receptor-independent pathway (Pate et al., 1998).

Albeit the precise mechanisms by which antiglaucomatous agents lower IOP are only partly understood, recent studies suggest that induction of matrix-degrading MMPs and subsequent remodeling of the extracellular matrix appear to be critical for numerous glaucoma therapies, including topical PG analogs (Weinreb and Lindsey, 2002) and argon laser trabeculoplasty (Parshley et al., 1996). In an attempt to link the expression of COX-2 with that of MMPs and to provide evidence for further outflow-facilitating mediators induced upon treatment of NPE cells with R(+)-MA, the expression of selected MMPs and TIMPs was focused on in additional experiments. RT-PCR and Western blot analyses revealed an R(+)-MA-elicited induction of MMP-1, -3, -9 and TIMP-1 at the mRNA as well as at the protein level. On the other hand, the expression of MMP-2 and TIMP-2 remained unaltered.

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As compared to the MMP-1 mRNA and protein levels obtained after treatment of cells with R(+)-MA, the induction of TIMP-1 was minor, suggesting that the availability and subsequent action of newly synthesized MMPs is unlikely to be considerably impaired in NPE cells.

There is evidence supporting a function of COX-2 in MMP-9 and TIMP-1 expression by R(+)-MA. Accordingly, inhibition of COX-2 activity by NS-398 was associated with a profound reduction of R(+)-MA-induced MMP-9 and TIMP-1 expression at both the transcriptional and translational level. Induction of MMP-9 and TIMP-1 mRNA had a delayed onset and was not evident at early times of COX-2 mRNA formation. Moreover, the effect of R(+)-MA on MMP-9 and TIMP-1 expression was mimicked by PGE_2, a major COX-2 product. In case of R(+)-MA-induced MMP-1 and MMP-3, concomitant incubation with NS-398 was accompanied by a partial but not significant suppressive effect on the mRNA and protein levels of the enzymes, suggesting that COX-2-dependent PGs do not play a crucial role in these responses. Additional experiments using two other COX-2-inducing cannabinoids, the structurally similar AEA and the phytocannabinoid Δ^9-THC, revealed similar effects. As a matter of fact, both compounds induced the same MMP/TIMP spectrum as R(+)-MA both in the presence and absence of NS-398.

Even though our data clearly point forward a PG-dependent regulation of MMP-9 and TIMP-1, it is worthy to note that the induction of MMPs and TIMPs may cell- and stimulus-dependently occur in either a PG-dependent or –independent manner. Accordingly, in the present study the COX-2 inhibitor NS-398 did not significantly interfere with R(+)-MA-induced MMP-1 expression, whereas our recent data have shown that MMP-1 expression elicited by the IOP-lowering $PGF_{2\alpha}$ analog latanoprost is inhibited by concomitant inhibition of COX-2 activity (Hinz et al., 2005). The reasons for these differential findings in the same cell type require further investigation. However, diverse signaling pathways elicited by cannabinoids and latanoprost as well as different kinetics and degrees of COX-2 induction

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and subsequent PG synthesis may provide a possible explanation concerning this matter. Interestingly, similar results have been published by Zhang et al. (1998) who showed a PGE_2-dependent induction of monocyte MMP-9 and TIMP-1 by lipopolysaccharide, whereas in the same cells the expression of MMP-9 and TIMP-1 by a mixture of cytokines occurred in a PGE_2-independent manner. In another study, unequal effects of MAP kinases have been proposed to explain agonist- and cell type-dependent regulation of MMPs. Thus, Lai et al. (2003) found that p38 MAPK regulates MMP-1 mainly through a PGE_2-dependent pathway, whereas p42/44 MAPK-mediated MMP-1 and MMP-9 production involves the activation of additional MMP promoter sites through a PGE_2-independent mechanism.

The results of this study imply the NPE as a possible target of cannabinoids in facilitating humor outflow. As the NPE is the primary source of aqueous humor, MMPs synthesized by these cells and secreted into aqueous humor subsequently, could theoretically be involved in the degradation of extracellular matrix of both uveoscleral and trabecular tissues. MMP-1, also referred to as interstitial collagenase, efficiently targets a specific site found in the fibrillar collagen types I and III (Jeffrey, 1998). In case of the uveoscleral pathway, collagen types I and III have been described as the major components of the fibrillar collagen in the extracellular matrix of the interstitial spaces among ciliary muscle fiber bundles (Rittig et al., 1990; Weinreb et al., 1994). MMP-3 efficiently cleaves specific sites found in collagen types IV, IX, XI, as well as in fibronectin and aggrecan core protein (Nagase, 1998). MMP-9 typically degrades the basement membrane constituent, collagen IV, and other matrix proteins (Van den Steen et al., 2002). Induction of MMP-1, -3 and -9 has been proposed to be involved in PG-stimulated uveoscleral outflow (Lindsey et al., 1996; Weinreb and Lindsey, 2002). With respect to the trabecular outflow facility, induction of MMP-3 and -9 has been observed during laser trabeculoplasty (Parshley et al., 1995, 1996). Likewise, Bradley et al. (1998) using a perfused human anterior segment organ culture demonstrated a more than 50% increase in outflow facility when purified MMP- 2, -3 and -9 was present in the perfusion

medium.

Apart from the clarification of the signaling events elicited by R(+)-MA and other cannabinoids in human NPE cells, our study extends a recent investigation on the role and function of intraocular COX-2. In this study COX-2 has been demonstrated to be constitutively expressed in the NPE of the ciliary body, but not in the NPE of patients with end-stage POAG (Maihöfner et al., 2001). Moreover, significantly minor PGE_2 levels were determined in aqueous humor of patients with POAG or steroid-induced glaucoma as compared to cataract patients (Maihöfner et al., 2001). On the basis of the results of the present study, it is therefore conceivable that induction of intraocular COX-2 and outflow-facilitating PGs is involved in the antiglaucomatous action of certain cannabinoids.

In summary, we have demonstrated for the first time that R(+)-MA and other cannabinoids are capable of activating the production of a broad spectrum of outflow-facilitating mediators, comprising PGE_2 and different MMPs, in human NPE cells. Our results further imply a contribution of COX-2-dependent PGs to the induction of MMP-9 and TIMP-1 by R(+)-MA. In the light of our investigations, activation of COX-2 and MMP expression could be an important mechanism by which R(+)-MA and other cannabinoids exert their IOP-lowering and antiglaucomatous action.

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FIGURE LEGENDS

Figure 1

Time-course (A) and modulation of R(+)-MA-induced PGE_2 synthesis by the selective COX-2 inhibitor NS-398 (B) in human NPE cells. Cells were incubated with R(+)-MA (10 μM) or its vehicle for the indicated times (A) or for 24 h (B) in the presence or absence of NS-398 (10 μM). Basal PGE_2 levels determined in supernatants of cells treated with vehicle for 24 h were 0.0035 ± 0.0002 μM (n = 3). Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± S.E.M. of n = 3 experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *vs*. corresponding vehicle control (Student´s *t*-test); $^{\#\#}P < 0.01$, *vs*. R(+)-MA (Student´s *t*-test).

Figure 2

Induction of COX-2 expression by different cannabinoids in human NPE cells.

(A,B) Induction of COX-2 mRNA expression by R(+)-MA. Cells were incubated with R(+)-MA (A: 10 μM, B: 0.1-10 μM) or its vehicle for the indicated times (A) or for 8 h (B). Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± S.E.M. of n = 3-6 experiments. **P* < 0.05, ***P* < 0.01, *vs*. corresponding vehicle control (Student´s *t*-test).

(C) Effect of R(+)-MA on COX-2- and COX-1 protein expression. Cells were incubated with R(+)-MA (10 μM) or its vehicle for 48 h. Results of the Western blots are representative of three experiments with similar results.

(D) Effect of AEA and Δ^9-THC on COX-2 protein expression. Cells were incubated with AEA (10 μM) or Δ^9-THC (10 μM) for 24 h. Results of the Western blots are representative of three experiments with similar results.

Figure 3

Time-course of R(+)-MA-induced phosphorylation of p38 (A) and p42/44 MAPKs (B) in human NPE cells. Cells were incubated with R(+)-MA (10 µM) for the indicated times. Activation of p38 and p42/44 MAPKs was analyzed by Western blotting using phospho-p38 MAPK and phospho-p42/44 MAPK antibodies, and antibodies against the non-phosphorylated forms as internal standards, respectively. Results are representative of three experiments with similar results.

Figure 4

Effect of inhibitors of MAPK pathways (A) and antagonists of cannabinoid and TRPV1 receptors (B) on R(+)-MA-induced COX-2 expression in human NPE cells. Cells were incubated with R(+)-MA (10 µM) or its vehicle in the presence or absence of SB203580 (p38 MAPK inhibitor; 1-30 µM), PD98059 (inhibitor of p42/44 MAPK activation; 1-30 µM), AM-251 (CB_1 receptor antagonist; 1 µM), AM-630 (CB_2 receptor antagonist; 1 µM) and capsazepine (TRPV1 receptor antagonist; 1 µM) for 8 h. All inhibitors were added 1 h prior to R(+)-MA to cell cultures. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± S.E.M. of n = 3 experiments. $^{*}P < 0.05$, $^{***}P < 0.001$, *vs.* corresponding vehicle control (Student´s *t*-test); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, *vs.* R(+)-MA (Student´s *t*-test).

Figure 5

Time-dependent effect of R(+)-MA on MMP-1, -2, -3, -9, TIMP-1 and -2 mRNA expression in human NPE cells. Cells were incubated with R(+)-MA (10 µM) for the indicated times. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test

substance. Values are means ± S.E.M. of n = 3-6 experiments. *P < 0.05, **P < 0.01, ***P < 0.001, *vs.* corresponding vehicle control (Student´s *t*-test).

Figure 6

Effect of the selective COX-2 inhibitor NS-398 on MMP-1, -2, -3, -9, TIMP-1 and -2 mRNA expression in R(+)-MA-treated human NPE cells. Cells were incubated with R(+)-MA (10 µM) or its vehicle for 24 h in the presence or absence of NS-398 (10 µM). Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± S.E.M. of n = 12 (except TIMP-2: n = 9) experiments. **P < 0.01, ***P < 0.001, *vs.* corresponding vehicle control (Student´s *t*-test); $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, *vs.* R(+)-MA (Student´s *t*-test).

Figure 7

Effect of the selective COX-2 inhibitor NS-398 on MMP-1, -2, -3, -9, TIMP-1 and -2 protein expression in R(+)-MA-treated human NPE cells. Cells were incubated with R(+)-MA (10 µM) or its vehicle for 48 h in the presence or absence of NS-398 (10 µM). Results of the Western blots are representative of three experiments with similar results.

Figure 8

Effect of PGE_2 on MMP-2, -3, -9, TIMP-1 and –2 protein expression in human NPE cells. Cells were incubated with PGE_2 (0.1-1 µM) or its vehicle for 24 h. Results of the Western blots are representative of three experiments with similar results.

Figure 9

Time-dependent induction of MMP-9 (A) and TIMP-1 (B) mRNA expression by PGE_2 in human NPE cells. Cells were incubated with PGE_2 (1 µM) or its vehicle for the indicated times. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± S.E.M. of n = 6-7 experiments. **P* < 0.05, ***P* < 0.01, *vs.* corresponding vehicle control (Student´s *t*-test).

Figure 10

Effect of the selective COX-2 inhibitor NS-398 on MMP-1, -2, -3, -9, TIMP-1 and -2 mRNA expression in AEA- (A) and Δ^9-THC-treated (B) human NPE cells. Cells were incubated with AEA (10 µM) or Δ^9-THC (10 µM) in the presence or absence of NS-398 (10 µM) for 24 h. PGE_2 levels in supernatants and MMP/TIMP mRNA in cell lysates were measured using ELISA and real-time RT-PCR, respectively. Basal PGE_2 levels determined in supernatants of cells treated with vehicle for 24 h were 0.0055 ± 0.0005 µM (n = 4) (A) and 0.0020 ± 0.0003 µM (n = 3) (B), respectively. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means ± S.E.M. of n = 4-6 (A) or n = 3-6 (B) experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *vs.* corresponding vehicle control (Student´s *t*-test); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, *vs.* the respective cannabinoid (Student´s *t*-test).

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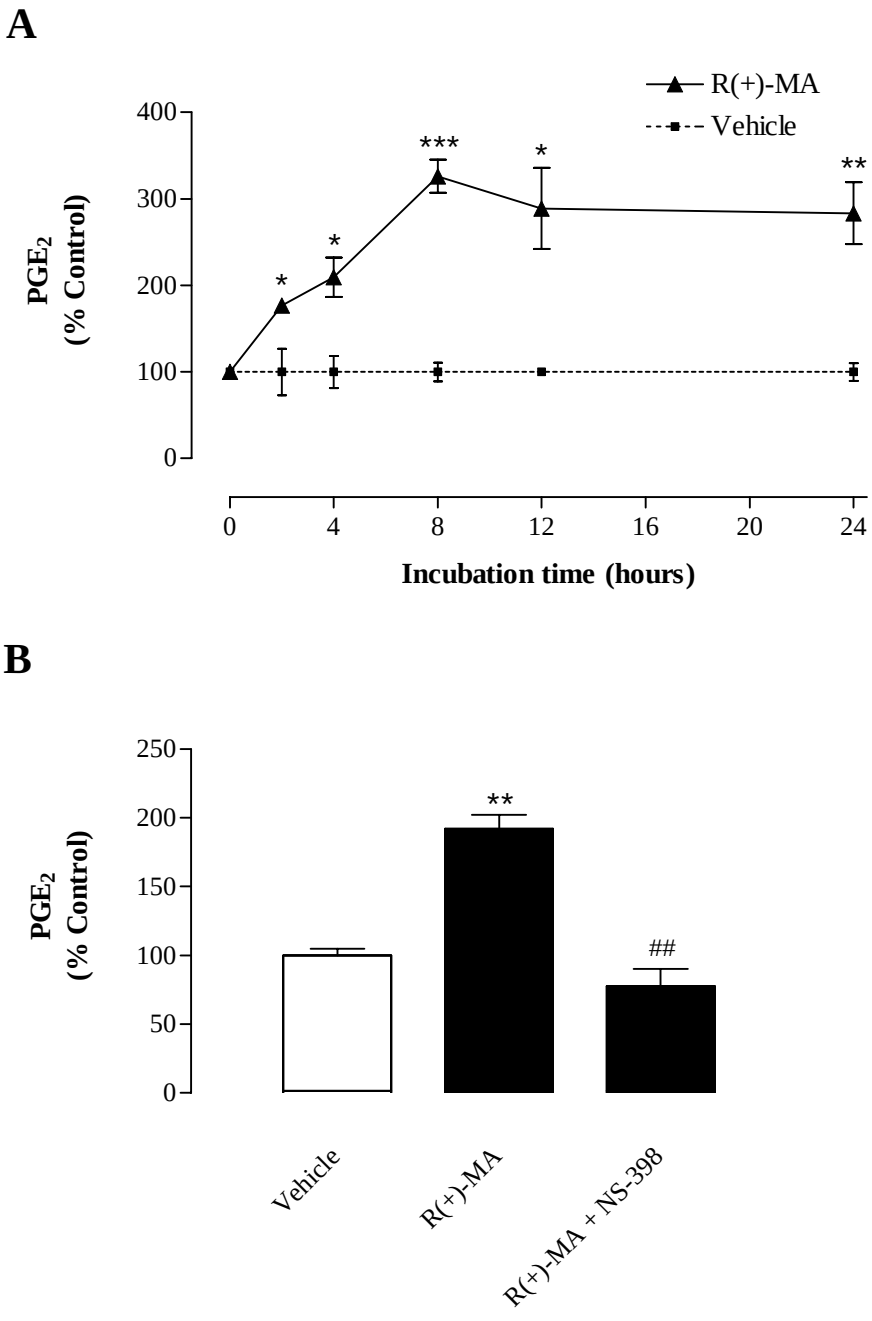

Fig. 1

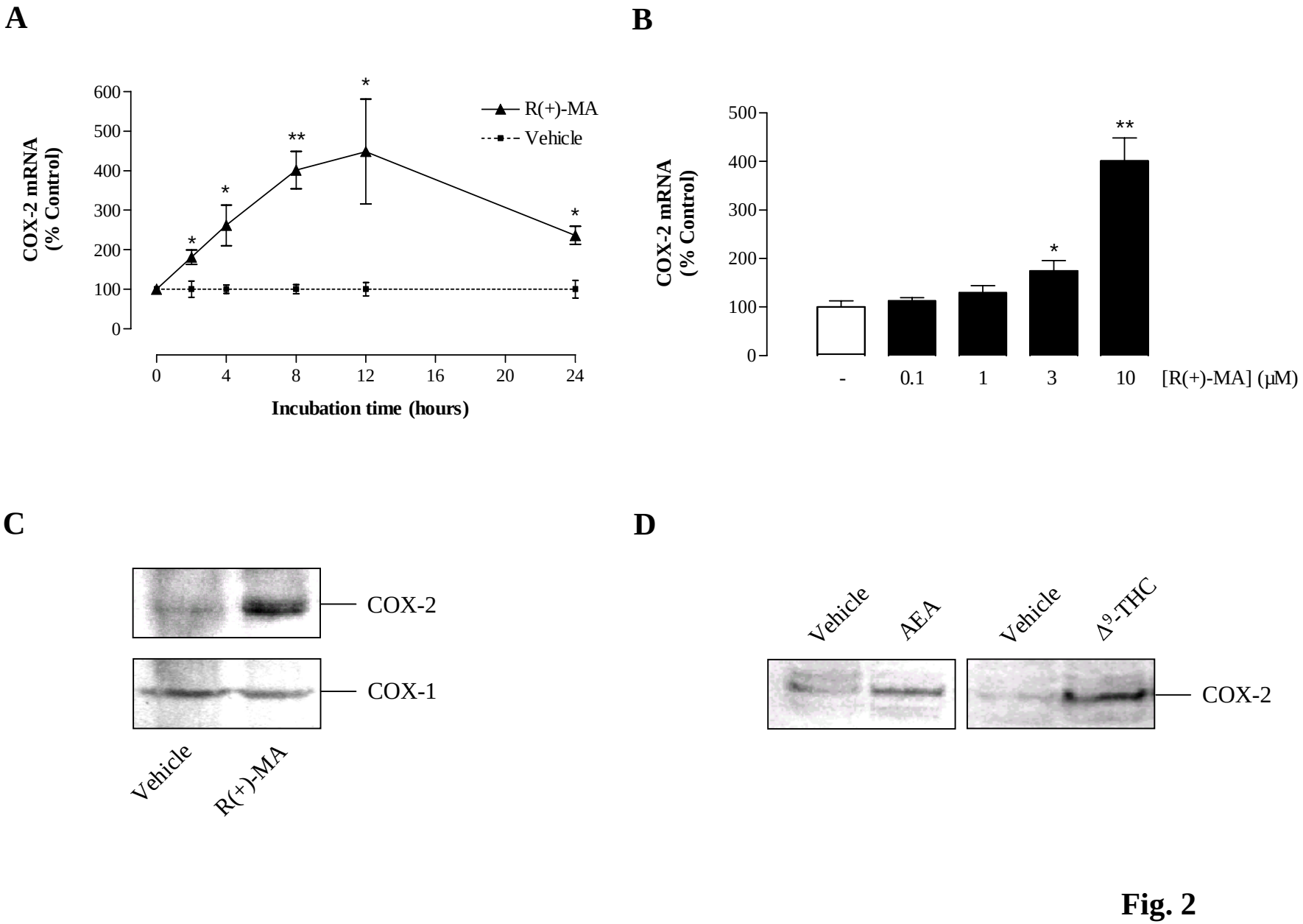

Fig. 2

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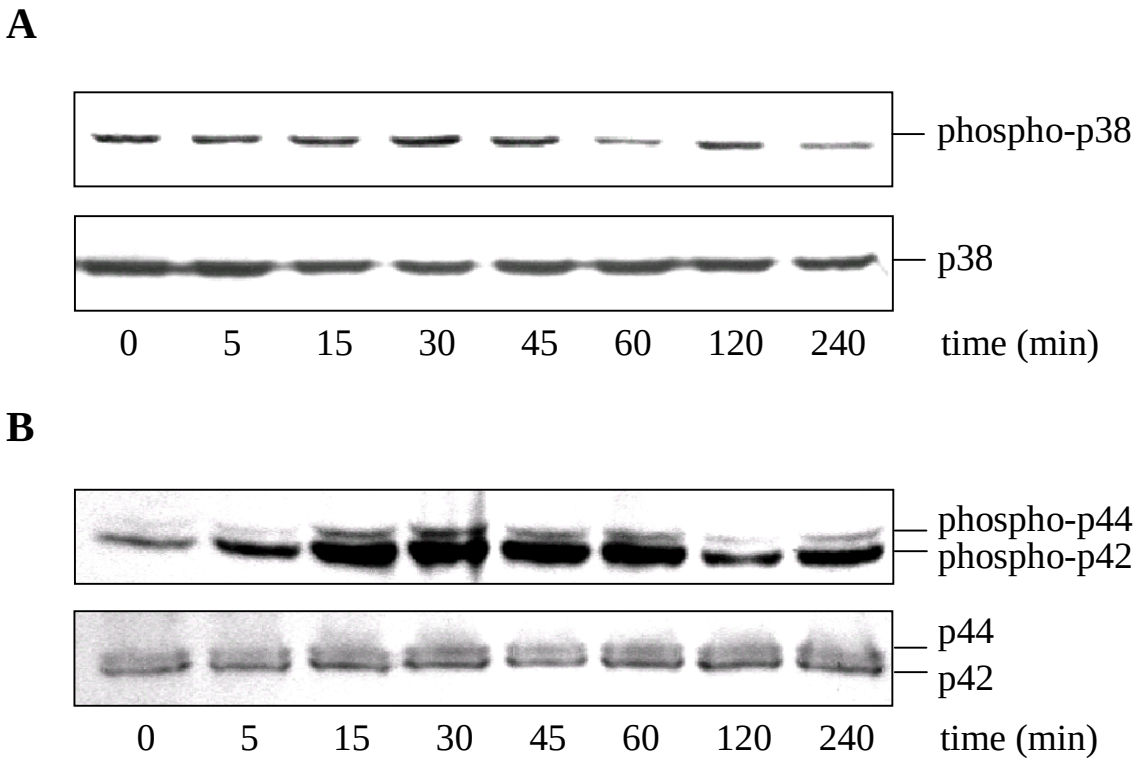

Fig. 3

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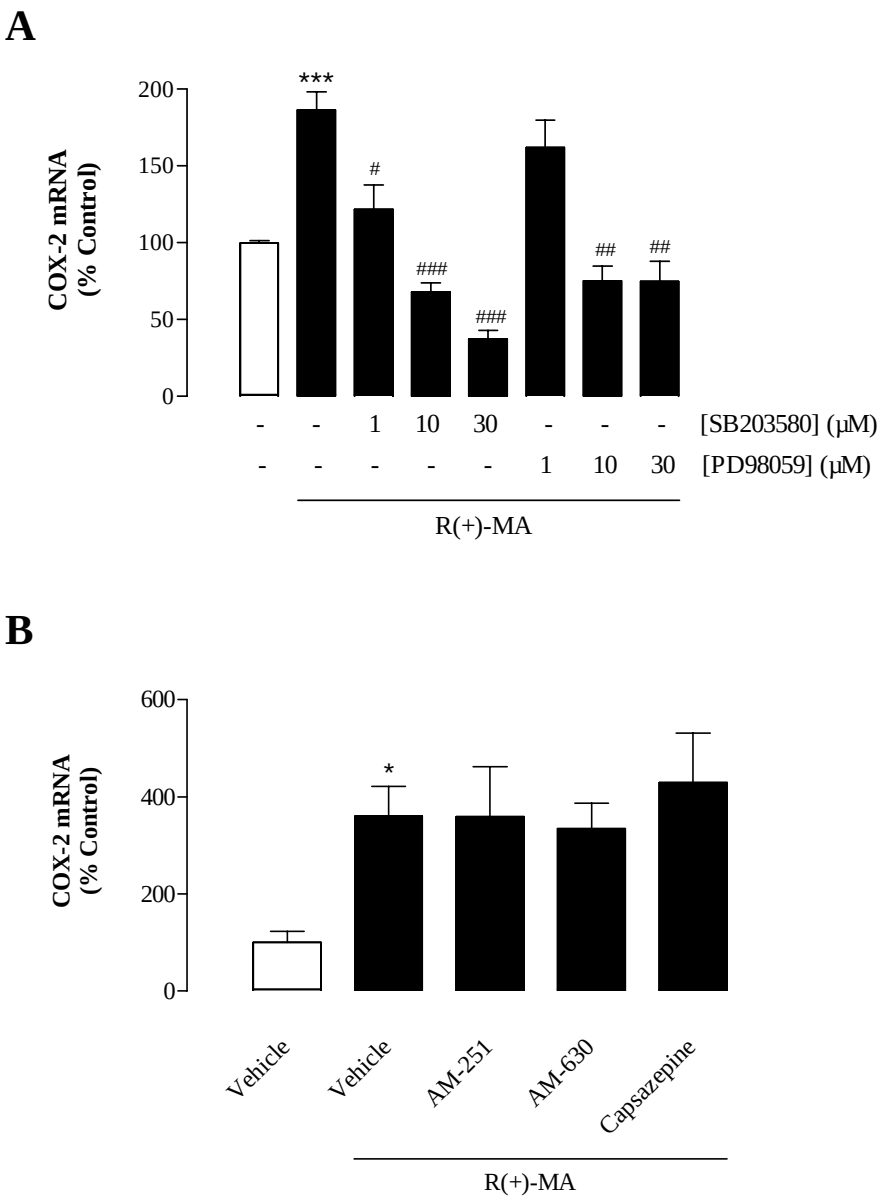

Fig. 4

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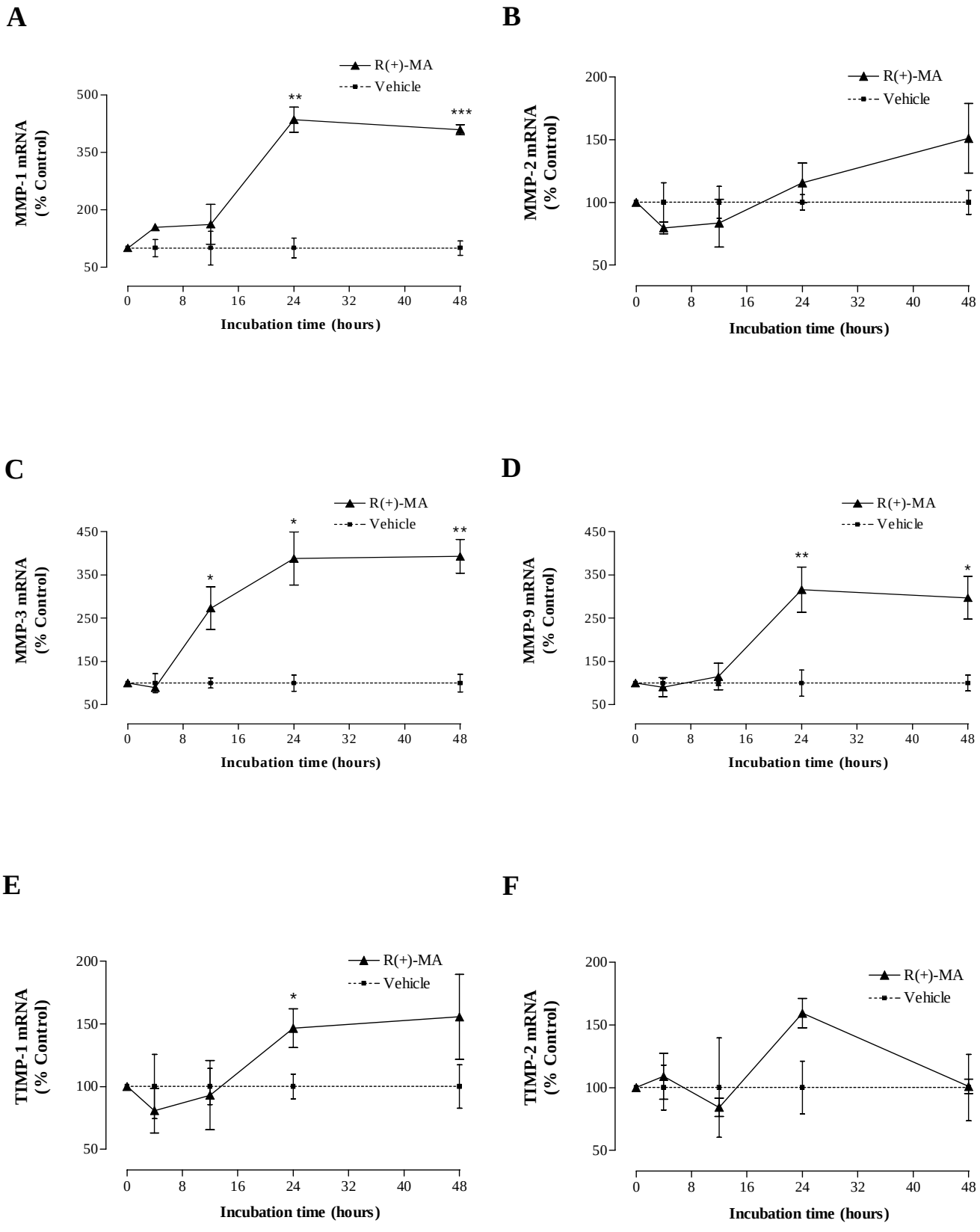

Fig. 5

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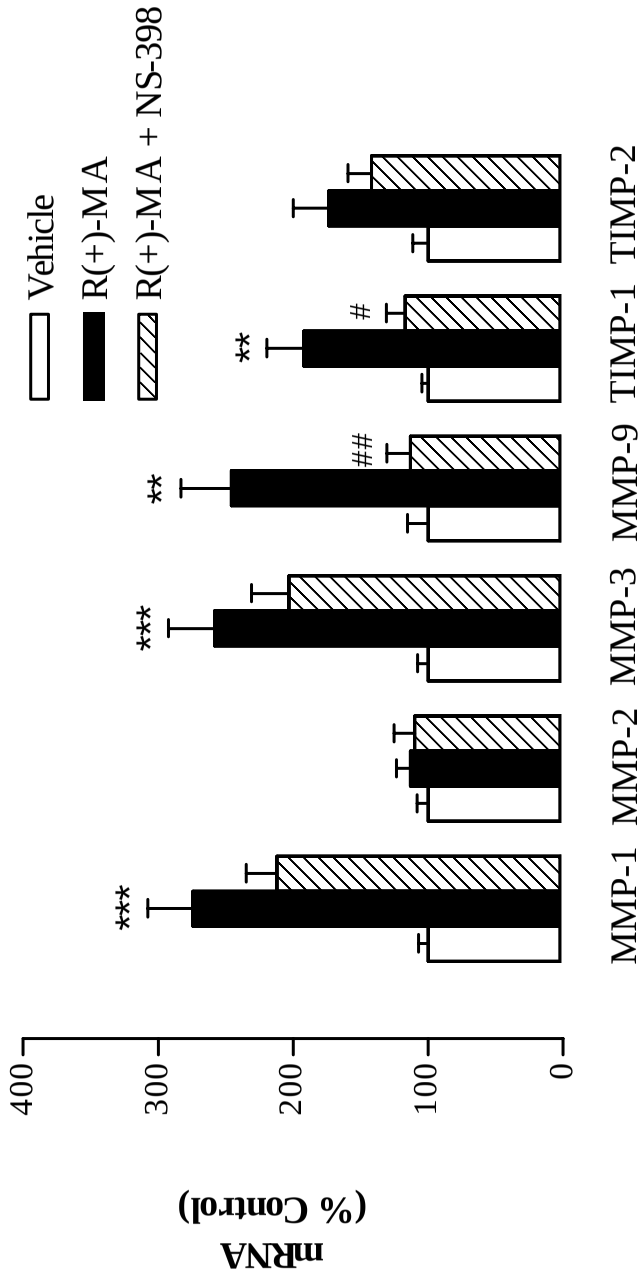

Fig. 6

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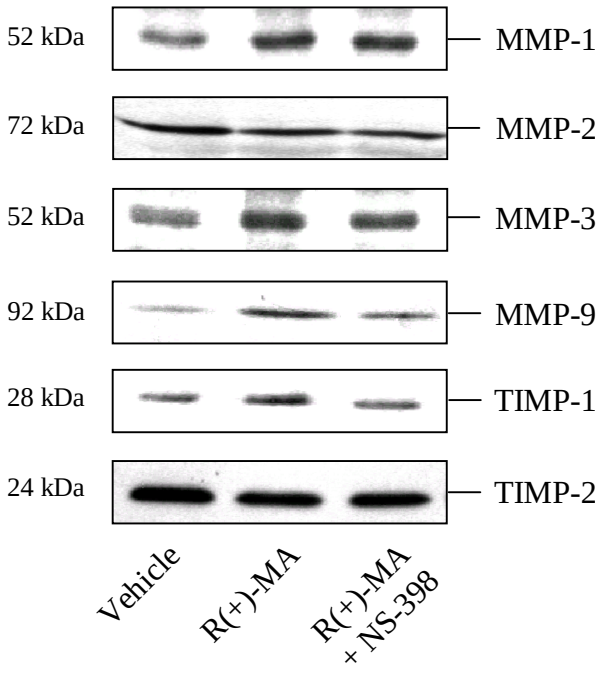

Fig. 7

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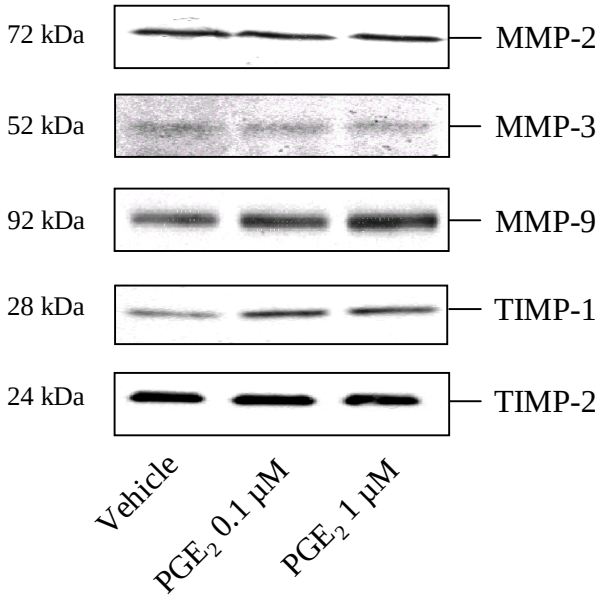

Fig. 8

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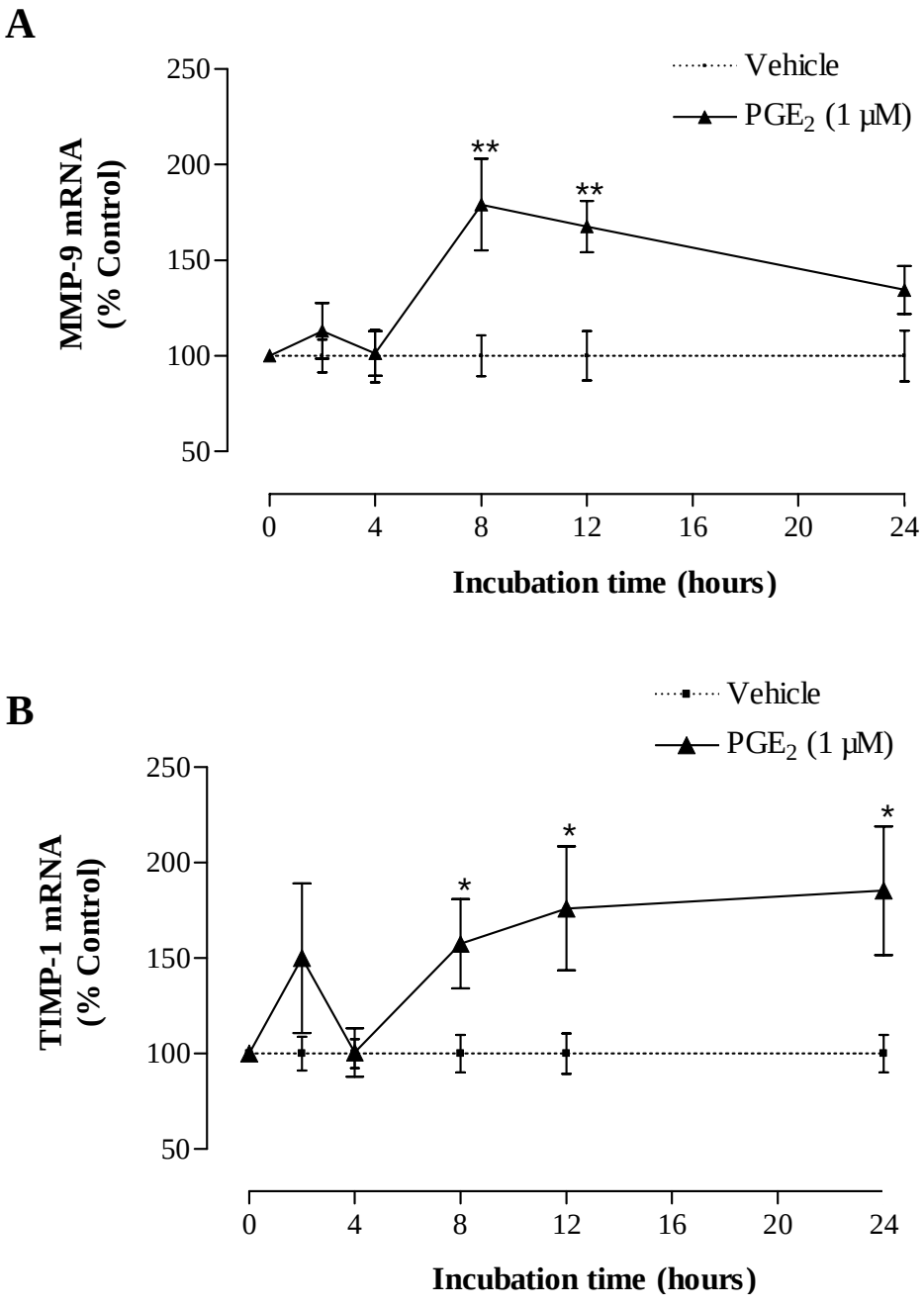

Fig. 9

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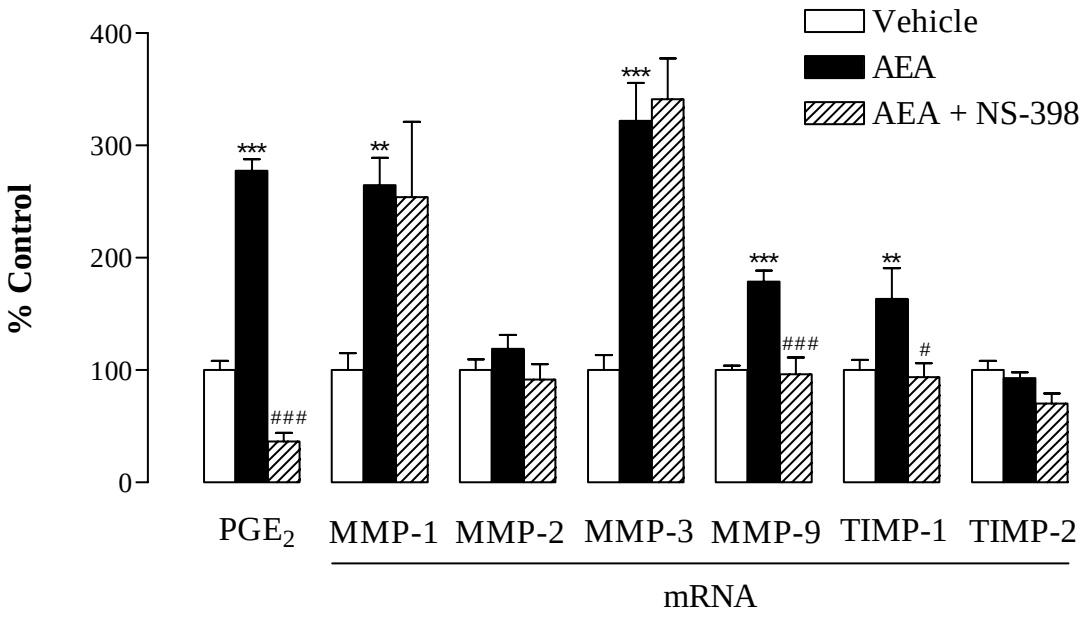
A



B

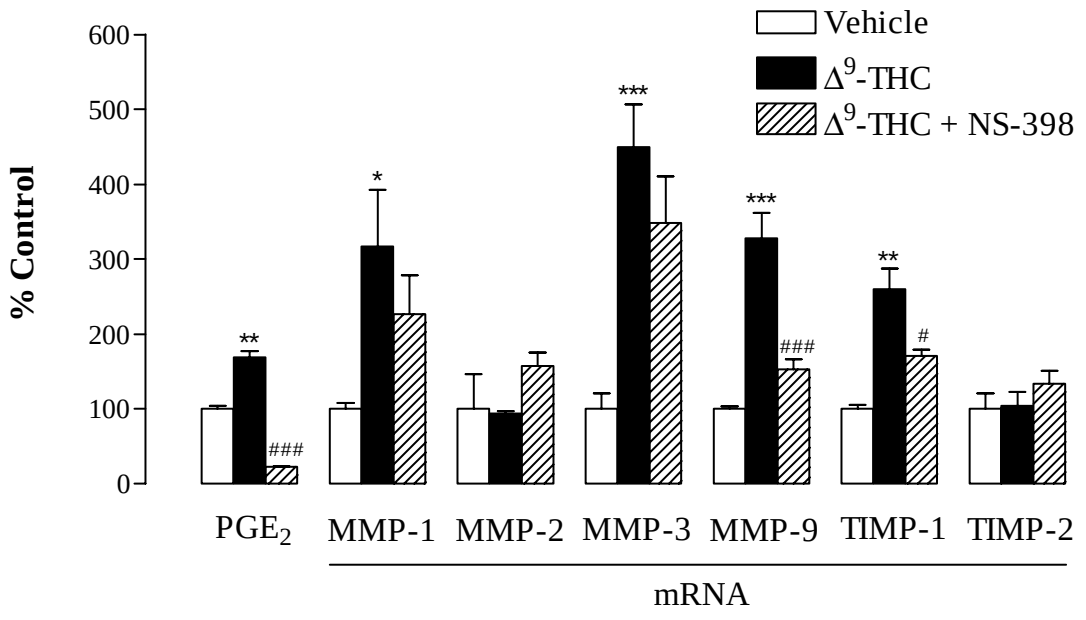


Fig. 10