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PGE₂ STIMULATES CANCER-RELATED PHENOTYPES IN PROSTATE CANCER PC3
CELLS THROUGH CYCLOOXYGENASE-2

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ABSTRACT

Cyclooxygenase (COX)-derived prostaglandin E₂ (PGE₂) affects many mechanisms that have been shown to play a role in carcinogenesis. Recently, we have found in androgen-independent prostate cancer PC3 cells that PGE₂ acts through an intracrine mechanism by which its uptake by the prostaglandin transporter PGT results in increased intracellular PGE₂ (iPGE₂), this leading to enhanced cell proliferation, migration, invasion, angiogenesis and loss of cell adhesion to collagen I). These iPGE₂-mediated effects were dependent on hypoxia-inducible factor 1- α (HIF-1 α), whose expression increased upon epidermal growth factor receptor (EGFR) transactivation by a subset of intracellular PGE₂ receptors. Here, we aimed to study in PC3 cells the role of COX in PGE₂ pro-tumoral effects and found that they were prevented by inhibition of COX-2, which highlights its crucial role as a mediator of PGE₂. Treatment with exogenous PGE₂ determined a transcriptional increase in COX-2 expression, which was abolished by genetic or pharmacologic inhibition of PGT. PGE₂-induced increase in COX-2 expression and, thereby, in transcriptional increase in HIF-1 α expression, was due to EGFR activation leading to activation of PI3K/Akt, Erk1/2, p38 and MSK-1. Collectively, the data suggest that EGFR-dependent COX-2 up-regulation by a novel positive feedback loop triggered by iPGE₂ underlies the intracrine pro-tumoral effects of PGE₂ in PC3 cells. Therefore, this feedback loop may be relevant in prostate cancer for the maintenance of PGE₂-dependent cancer cell growth through amplifying the activity of the COX-2 pathway.

1. Introduction

Chronic inflammation is a risk factor for the development and progression of prostate cancer (PC) (reviewed in [1]). In PC it has been found overexpression of cyclooxygenase-2 (COX-2) and inflammatory mediators such are cytokines and chemokines [2-4]. COX-1 and COX-2 are isoforms of cyclooxygenase that convert arachidonic acid to prostaglandins (PGs). COX-1 is constitutively expressed whereas inflammation is associated to the induction of COX-2. Prostaglandin E₂ (PGE₂) has been found at increased levels in PC as well as in other malignant neoplasms such are lung cancer, breast cancer and colon cancer [5,6]. Proliferation, migration, invasion, apoptosis, and angiogenesis are targets of the carcinogenic effects of PGE₂ in PC and other types of cancer [7-11]: it has been shown in experimental PC that treatment with non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX (and thereby the production of PGE₂), induce apoptosis, inhibits cell proliferation and decreases metastasis [12,13]. We have found that several pro-tumoral actions of PGE₂ in PC3 cells depend on its transport to the inside the cells and further activation by this intracellular PGE₂ of a subset of intracellular EP receptors [14,15]. This is a non-canonical intracrine mechanism of PGE₂ action in which the prostaglandin transporter (PGT, responsible for most PGE₂ internalization) mediates the influx of PGE₂ [16,17]. In PC3 cells, intracellular PGE₂ (iPGE₂), inhibits cell adhesion and stimulates cell proliferation, migration, invasion and in vitro angiogenesis [14,15] and these effects were dependent on hypoxia-inducible factor-1 α (HIF-1 α). The expression and activity of HIF-1 α increased upon epidermal growth factor receptor (EGFR) transactivation by intracellular EP receptors [15]. It has been previously

described in human cancer cells from prostate, breast and colon, that PGE₂ induces COX-2 [18,19], which suggests that the intracrine effects of PGE₂ in PC3 cells might be mediated by the induction of COX-2. In the present study, we show that this is indeed the case because inhibition of COX-2 resulted in prevention of HIF-1 α up-regulation and of all the pro-tumorigenic intracrine effects of PGE₂ in PC3 cells. We also show that PGE₂ triggers an EGFR-dependent positive feedback loop of COX-2 up-regulation, which might contribute to the up-regulation of COX-2 during prostate cancer as well and, thereby, to the maintenance of PGE₂-dependent cancer cell growth through the continuous synthesis of PGE₂.

2. Materials and methods

2.1. Reagents and antibodies

PGE₂, bromosulphophthalein (Bs), AG1478, 5'-Br-2'-deoxyuridine (BrdU), actinomycin D (Act.D), cycloheximide (CHX), 3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole (YC1), nimesulide, LY294002, PD98059, H89, PF04419948, SB203598 and type I-collagen were purchased from Sigma (St. Louis, MO, USA). Celecoxib and diclofenac were acquired from Cayman Chemical (Ann Arbor, MI, USA) and PP2 was purchased from Calbiochem Millipore (Darmstadt, Germany). PGT siRNA or MSK-1 siRNA, and scramble were from Santa Cruz Biotechnology (Temecula, CA, USA) and Applied Biosystem (Foster City, CA, USA), respectively.

Antibodies were from the following sources: anti-COX-2 and anti-PGE₂ were acquired from Abcam (Cambridge, UK). Anti- β -actin was purchased from antibodies Sigma (St. Louis, MO, USA). Anti-PGT, α -mouse-Alexa-Fluor®488 and α -rabbit-Alexa-Fluor®488 antibodies were from Invitrogen (Eugene, Oregon USA). Anti-HIF-1 α antibody and anti-BrdU antibodies were acquired from BD Biosciences (Palo Alto, CA, USA). Anti-COX-1, anti-phospho EGFR and anti-phospho MSK-1 antibodies were purchased from Santa Cruz Biotechnology (Temecula, CA, USA). Anti-phospho Src and anti-rabbit IgG peroxidase conjugate antibodies were from Calbiochem Millipore (Darmstadt, Germany). Anti-phospho Akt, anti-phospho Erk1/2 and anti-phospho p38 antibodies were acquired from Cell Signaling (Danvers, MA, USA).

All other reactives were purchased from Sigma (St. Louis, MO, USA).

2.2 Cell Culture

The human prostate cancer cell line PC3 was from purchased to American Type Culture Collection (Manassas, VA). Cells were grown in sterile conditions and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin /streptomycin/amphoterycin B from Life Technologies (Barcelona, Spain). The culture was performed in a humidified 5% CO₂ environment at 37 °C. After the cells

reached 70–80% confluence, they were washed with PBS, detached with 0.25% trypsin/0.2% EDTA and seeded at 30,000–40,000 cells/cm². The culture medium was changed every 3 days.

2.3. Cell proliferation assay with 5'-Br-2'-deoxyuridine (BrdU)

DNA synthesis was assessed by BrdU uptake. PC3 cells were placed in 24-well plates (6 x 10⁴ cells/glass coverslip) and they were grown for 24 h. Then, they were maintained for 24 h in serum free RPMI-1640 and treated as described later. Afterwards, the cells were pulsed with 10 μM BrdU during the last 16 h of incubation. Then, they were fixed with 2% paraformaldehyde for 15 min, DNA was partially denatured by incubation with 2 M HCl for 20 min, after which the cells were incubated with 0.1 M sodium borate for 2 min at room temperature. Then, the cells were permeabilized with PBS containing 0.2% Triton x-100 (pH 7,4) for 5 min and 3% bovine serum albumin and were washed three times with PBS. Cells were incubated at 37°C with anti-BrdU monoclonal antibody (1:50) for 1 h. Then, they were then incubated at 37°C with α-mouse-Alexa-Fluor® 488 (1:2000) for 1 h in the darkness. Cell nuclei were counterstained with Prolong Gold antifade Reagent with DAPI (Invitrogen Eugene, OR). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany). To estimate DNA synthesis, the percentage of BrdU-positive nuclei was determined through manual count in five fields in a blind manner. Quantitative analysis of BrdU-positive cells among the sorted cells was performed by counting the positive nuclei in total cells per field (total cells were visualized by DAPI-positive nuclei).

2.4. Cell Adhesion Assay.

The cell adhesion to collagen was evaluated as follows: cultured cells were detached by trypsinization, resuspended in serum-free RPMI-1640 medium (2.5 x 10⁵ cells/ml) and treated as stated in the results section. Then cells were plated in 96-well plates pre-coated with 0.1 ml of 0.0167 g/100 ml acetic acid and type-I collagen from Sigma (St. Louis, MO) and incubated at 37°C for 30 min. The non-adherent cells were washed out with PBS, and the number of cells that adhered to collagen was assessed by MTT assay. The independent experiments were run in three times.

2.5. Cell migration assay

PC3 cells were seeded in 24-well plates (3 x 10⁵ cells per well). At 24 h after seeding, the monolayer cells were manually scratched with a pipette yellow tip to create extended and definite scratches in the center of the dishes with a bright and clear field (~ 2 mm). Cells were treated as indicated in the results section and the narrowing of the wound by migrating cells was monitored by measuring the width in microphotographs. Three representative fields of each monolayer wound were captured using a Nikon Diaphot 300 inverted microscopy camera (10x) up to 30 h. Monolayer wound areas of untreated samples were averaged and assigned a value of 100.

2.6. Cell invasion assays

For the invasion assays, we used transwell polycarbonate filters (8- μ m pore size, Corning Costar, Cambridge, UK), which were coated with 50 μ l of Matrigel Basement Membrane Matrix (BD Biosciences Bedford, MA, 1:10 dilution with serum free media). Cells were harvested and resuspended in serum-free RPMI-1640 medium at a concentration of 5×10^5 cells/ml. Cells in suspension were treated as indicated in the results section and then 0.2 ml of cell suspension and 0.6 ml complete medium were added to the upper and lower chamber, respectively. Cells were incubated for 48 h to allow them to colonize the lower chamber. Invasive Cells were fixed with methanol, stained with Giemsa and counted in four different fields in a Nikon inverted microscope camera (20x).

2.7. Western blot analysis

PC3 cells were split into six-well plates at a density of 3×10^5 cells/well and incubated for 24 h before treatment. Afterwards immunoblotting was performed essentially as described previously [20]. In short, cell lysates were prepared and measured for protein content using the Bradford assay. Approximately 35 μ g of protein was electrophoresed 10% SDS–polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with: anti-COX-2 (1:1000), anti-COX-1 (1:1000), anti-HIF-1 α (1:1000), anti-phospho EGFR (1:500), anti-PGT (1:1000), anti-phospho- c-Src (1:1000), anti-phospho-Erk1/2 (1:1000), anti-phospho-MSK-1 (1:1000), anti-phospho-Akt (1:1000), anti-phospho-p38 (1:1000). After, membranes were incubated at room temperature for 1 h, with the corresponding secondary antiserum (1:4000). To ensure equal loading of proteins, the membranes were stripped and re-probed with anti- β -actin antibody. The signals were detected with enhanced chemiluminescence reagent (Amersham Healthcare, Buckinghamshire, England). Quantification of band densities was performed using Quantitative One Program (Bio-Rad, Alcobendas, Spain)

2.8. Promoter COX-2 reporter gene assay

Cells were split into six-well plates at a density of 3×10^5 cells/well. 24 h later, the cells were cotransfected with 0.5 μ g/well luciferase plasmid COX-2-Luc, the human COX-2 luciferase reporter construct phPES2 containing the promoter fragments -327 to +59 was a gift from Dr Hiroyasu Inoue (Nara Women's University, Nara, Japan), and 0.5 μ g/well of the *R. reniformis* luciferase reporter pRL-CMV using lipofectamine (Invitrogen, CA). Transfected cells were next incubated with complete growth medium for 24 h, and then they were treated as indicated in the results section. Afterwards cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI). The data were normalized against the *R. reniformis* luciferase activity.

2.9. Transient transfection with siRNA

For PGT and MSK-1 inhibition, we used either PGT siRNA sc-78211 or MSK-1 siRNA sc-35977 (Santa Cruz Biotechnologies) respectively, and scramble siRNA AM4637 (Applied Biosystems) as a control. PC3 cells at 80% of confluence were transfected with 100 nM siRNA PGT, or 100 nM siRNA scramble. According to the manufacturer's protocol, we used Lipofectamine 2000 (Invitrogen) to get the transfection. Transfected cells were next incubated with complete growth medium for 24 h, and then they were treated as indicated in the results section. Afterwards cells were harvested and protein expression was measured by Western blot analysis.

2.10. RNA isolation and RT-PCR

PC3 cells were seeded in 6-well plates (3×10^5 cells/well) grown for 24 h and treated as described later. Total cellular RNA was isolated with TriReagent reagent from Sigma (St. Louis, MO) according to the instructions of the manufacturer. One microgram of total RNA was reverse-transcribed using 50 pmol of hexamer random primer and 0,5µl PrimeScript RT enzyme supplemented with 25 pmol of oligo dT primers from Takara (Shiga, Japon). RT conditions were: denaturation at 94°C for 10 min, followed by the reaction of RT at 37°C for 1 h and then a final reaction of 5 min at 95°C. Two microliters of RT reaction were then PCR-amplified with specific primers for COX-2 (sense 5'- GAT ACT CAG GCA GAG ATG ATC TAC CC-3'; antisense 5'-AGA CCA GGC ACC AGA CCA AAG A -3'), HIF-1α (sense 5'-GAA AGC GCA AGT CCT CAA AG-3'; antisense 5'- TGG GTA GGA GAT GGA GAT GC-3') or GAPDH (sense 5'-CAA GGG CAT CCT GGG CTA C-3'; antisense 5'- TTG AAG TCA GAG GAG ACC ACC TG-3'). PCR conditions were as follows: 94°C for 5 min (denaturation), followed by 37-40 cycles of 95°C 1 min, 54 °C 1 min and then a final cycle of 1 min at 72°C. The signals were normalized with GAPDH gene expression level. The PCR products were separated by electrophoresis and visualized in 2% agarose gels.

2.11. Immunofluorescence analysis

PC3 cells were placed in 24-well plates (104 cells/glass coverslip) were treated as indicated in the results section. Then, they were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min, washed with PBS, blocked with 4% bovine serum albumin for 1 h at room temperature and incubated overnight at 4°C with anti-PGE₂ antibody (1:50 dilution). Cells were then incubated at 37°C with α-rabbit-Alexa-Fluor® 488 (1:2000) for 1 h in the darkness. Coverslips were then washed and mounted with ProLong Gold antifade Reagent with DAPI (Invitrogen Eugene, OR). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany). Images were analysed using ImageJ software (NIH).

2.11. Statistical analysis

Each experiment was repeated at least three times. The results are expressed as the mean \pm standard deviation (SD). They were subjected to one-way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1. PGE₂ increases PC3 cell proliferation, migration, invasion and HIF-1 α expression, and decreases PC3 cell adhesion to collagen I in a COX-2-dependent manner.

We have previously found that PGE₂ increases PC3 cell proliferation, migration, invasion and decreases PC3 cell adhesion to collagen I. These effects are very relevant in the pathogenesis of cancer. First, unchecked proliferation is a hallmark of cancer cells that commonly exhibit increased proliferation when compared to normal cells. Second, the most threatening feature of malignancy in cancer is the potential for invasion and metastases: cancer cell detachment is the initial event in metastases formation of carcinomas and tumor cells often show a decrease in cell–cell and/or cell–matrix adhesion. Besides cell detachment, the migratory activity of cancer cells also contributes to metastasis. Taking into account these considerations and our aim of studying in PC3 cells the role of COX in PGE₂ effects, we first asked whether inhibition of COX prevented pro-tumorigenic effects of PGE₂ such as cell proliferation, detachment, migration, invasion and HIF-1 α up-regulation. To this end, PC3 cells were pre-incubated with diclofenac (to inhibit both COX-1 and COX-2 isoenzymes) or with celecoxib (to inhibit COX-2 activity; in the case of HIF-1 α the effect of a second COX-2 inhibitor, nimesulide, was also assessed), before being exposed to PGE₂. As shown in Figure 1 both NSAIDs prevented the effects of PGE₂ on cell proliferation (without affecting cell survival as assessed by annexin V/propidium iodide staining, results are not shown), adhesion to collagen I, migration, invasion and HIF-1 α up-regulation (see Fig. S.1 for quantification of the Western blot analysis), which indicated that these PGE₂ effects are dependent on COX activity. Worth to mentioning, all NSAIDs used inhibited the basal expression of HIF-1 α (results are not shown, except for diclofenac in Fig. 1 3, right). This fact is in good agreement with previous studies [21] and indicates that COX-2 activity plays a critical role in the regulation of HIF-1 α expression. On the other hand, the fact that the inhibitory action of diclofenac was not stronger than that of celecoxib suggests that COX-2 is the COX isoenzyme which specifically mediates the studied effects of PGE₂ on PC3 cells.

3.2. PGE₂ up-regulates the expression of its own synthesizing enzyme COX-2 in a PGT-dependent manner.

PGE₂ induces COX-2 in human colon cancer cells, breast cancer cells and PC cells, although the latter only has been analyzed at the mRNA level but not at the protein level of COX-2 regulation [18,19]. Since PGE₂-induced COX-2 up-regulation might contribute to the pro-tumoral actions of PGE₂ in PC3 cells, thereby explaining the inhibitory effect of celecoxib on these actions, we studied the effect of PGE₂ in the expression of COX isoenzymes. Our results indicated that COX-2 expression, but not COX-1 expression, increased over the time (Fig. 2 a, upper panel and Fig. S.2 a, upper panel). In fact, COX-2 behaved as an early-response protein because its expression increased 30 min after treatment with PGE₂ (results are not shown). Parallely to the increase in COX-2 expression, there was a diclofenac-sensitive rise in the levels of iPGE₂ (Fig. 2 a middle panel). Further mechanistic studies (Fig. 2 a lower panel and Fig. S.2.a lower panel) showed that PGE₂-induced increase in COX-2 protein expression was prevented by the inhibitor of transcription actinomycin D and that treatment of PGE₂ determined an increase in COX-2 mRNA expression, these results indicating that induction of COX-2 mRNA contributed to the increase in COX-2 protein expression.

We have previously found that an iPGE₂-activated mechanism, requiring the previous transport of PGE₂ to the inside the cell, mediates the pro-tumorigenic effects of PGE₂ on PC3 cells [14]. Since COX-2 also mediates these effects (Fig.1), we reasoned that iPGE₂ might also be responsible for PGE₂-induced COX-2 up-regulation. In order to test this hypothesis, we assessed the effect of inhibiting PGT (i.e. the PGE₂ uptake transporter) on the up-regulation of COX-2 by PGE₂. Fig 2 b (upper panel) shows that iPGE₂ increased shortly after treatment with PGE₂ in a PGT-inhibitor sensitive manner and that, as expected, knockdown or pharmacological inhibition of PGT resulted in prevention of PGE₂-induced increase in both COX-2 expression (Fig. 2 b middle panel and Fig. S.2.b) and activity of a COX-2 promoter construct transfected in PC3 cells (Fig. 2 b lower panel). In summary, the results shown in Figure 2 indicate that PGE₂ up-regulates the expression of its own synthesizing enzyme COX-2 in a PGT-dependent manner.

3.3. c-Src-dependent activation of EGFR mediates the up-regulation of COX-2 by PGE₂.

In PC3 cells, PGE₂ augments the phosphorylation of EGFR [9] and we have previously found that the transactivation of EGFR by EP2 receptor and further EGFR-dependent increase in HIF-1 α expression, are critical events leading to the pro-tumorigenic effects of PGE₂ on PC3 cells [15]. In consequence, it was likely that EGFR mediated the up-regulation of COX-2 by PGE₂. We explored this possibility by assessing the prevention of PGE₂-induced increase in COX-2 expression by the inhibitor of EGFR activation, AG1478. The results shown in Fig. 3 a (and Fig. S.3.a) in which AG1478 prevented the increase in COX-2 protein and mRNA and in the activity of a COX-2 promoter construct transfected in PC3 cells, confirmed our hypothesis. Furthermore, treatment with PGE₂ resulted in increased phosphorylation of EGFR, which was prevented by the inhibitor of PGT transporter bromosulphophthalein (Fig. 3 b and Fig. S.3.b). The latter results confirmed that

activation of EGFR mediated the up-regulation of COX-2 by PGE₂ and highlighted the role in the mechanism of action of PGE₂ of its PGT-dependent uptake by PC3 cells.

Our previous studies have shown that c-Src mediates the transactivation of EGFR in human renal proximal tubular HK-2 cells treated with PGE₂ [22]. In order to assess the relevance of c-Src in the mechanism through which PGE₂ increases the expression of COX-2 in PC3, we studied the effect of c-Src inhibitor PP2 on PGE₂-induced EGFR phosphorylation and COX-2 up-regulation. Our results indicated (Fig. 3 c-d and Fig. S.3.c-d) that PP2 prevented both effects of PGE₂ and that c-Src phosphorylation increased upon treatment with PGE₂. These results indicate that c-Src mediates the phosphorylation of EGFR that leads to the up-regulation of COX-2 by PGE₂.

3.4. Erk1/2/p38/MSK-1 and PI3k/Akt pathways mediate the EGFR-dependent-increase in COX-2 expression upon treatment with PGE₂.

We have previously found in human renal proximal tubular HK-2 cells that PI3k/Akt and Erk1/2/p38/MSK-1 pathways mediate the EGFR-dependent increase in HIF-1 α upon treatment with PGE₂ ([22] unpublished results). Since PGE₂ also induces an EGFR-, COX-2-dependent increase in HIF-1 α expression in PC3 cells ([15] and Fig. 1). We studied the role of PI3k/Akt and Erk1/2/p38/MSK-1 pathways as possible mediators of the EGFR-dependent increase in COX-2 induced by PGE₂. To this end, we first studied whether pre-treatment with LY294002 and wortmaninn (inhibitors of PI3k), PD98059 (inhibitor of the MAPK kinase which is upstream of Erk1/2), SB203580 (p38 MAPK inhibitor), or H89 (MSK-1 inhibitor) or siRNA MSK-1 (given that H89 inhibits PKA too, we also inhibited MSK-1 through knocking it down) prevented the PGE₂-induced increase in COX-2 expression, which was confirmed by the results shown in the Figure 4 a and Fig. S.4.a. These inhibitors also prevented the increase in the activity of a COX-2 promoter construct in PC3 cells treated with PGE₂ (Fig. 4 a) Furthermore, treatment with PGE₂ resulted in phosphorylation of Akt, Erk1/2, p38, and MSK-1 (Fig. 4 b and Fig. S.4.b) (which is activated downstream of the ERK or p38 MAPK pathways in vivo [23], this effect being prevented by EGFR inhibitor AG1478 (Fig. 4 c and Fig. S.4.c) (of note, PGE₂-induced MSK-1 phosphorylation was prevented by PD98059 and SB203580, but not by LY294002 (Fig. 4 d and Fig. S.4.d), which indicates that the pathways PI3k/Akt and Erk1/2/p38/MSK-1 are independent).

Taken together, the results shown in Figure 4 indicate that PI3k/Akt and Erk1/2/p38/MSK-1 pathways mediate the EGFR-dependent-increase in COX-2 expression upon treatment with PGE₂.

3.5. PGE₂ increases transcriptionally the expression of HIF-1 α through an intracrine mechanism involving the same signaling pathways that mediate COX-2 up-regulation.

In PC3 cells, we have demonstrated that the pro-tumoral effects of PGE₂ are mediated by the BG-sensitive increase in HIF-1 α expression and activity [14]. However, we did not study the mechanisms and signaling pathways leading to HIF-1 α up-regulation. In order to address this issue, we first studied the mechanisms through which PGE₂ increases HIF-1 α expression. Previous studies indicate that up-regulation of HIF-1 α by PGE₂ in HK-2 cells is not due to an actinomycin-D insensitive increase in HIF-1 α protein stability –which is the canonical pathway of hypoxia-induced HIF-1 α accumulation [24-26]- but to an actinomycin D-sensitive, protein stability-independent mechanism [27]. To test if this was also the case in PC3 cells, we first analysed the effect of PGE₂ on HIF-1 α expression. As shown in Figure 5 a left and Fig. S.5.a left, PGE₂ determined an increase in the expression of HIF-1 α mRNA in PC3 cells. Furthermore, pre-incubation of PC3 cells with the inhibitor of transcription actinomycin D prevent the up-regulation of HIF-1 α protein in PGE₂-treated cells (Fig. 5 a right and Fig. S.5.a right). These results indicate the intervention of transcriptional mechanisms, but they do not rule out the hypothetical contribution of stabilization of HIF-1 α protein. This issue was addressed by analysing the effect of PGE₂ on in the turnover of HIF-1 α . To this end, we studied the time-course of HIF-1 α expression (Western blot analysis) in PC3 cells that were first incubated for 6 h under control conditions or with PGE₂ and then treated for 0-30 min with translation inhibitor cycloheximide. As shown in Fig. 5 a right, inset and Fig. S.5.a right, inset, HIF-1 α protein half-life under hypoxia was not modified by PGE₂. Altogether, these results demonstrate that transcriptional mechanisms are responsible for the increase in HIF-1 α expression induced by PGE₂.

Finally, we sought to confirm the relevance of PGE₂-induced increase in COX-2 expression in the up-regulation of HIF-1 α by PGE₂. To this end, we studied the effect on PGE₂-induced increase in HIF-1 α expression of the inhibitors of COX-2 up-regulation. Figure 5 b and Fig. S.5.b. shows that all the maneuvers that prevent the up-regulation of COX-2 by PGE₂ (i.e. genetic knockdown of PGT and MSK-1, and pharmacological inhibition of c-Src, EGFR, Erk1/2, p38, MSK-1 and PI3K), also prevented the up-regulation of HIF-1 α by PGE₂. These results highlight the relevance of PGE₂-induced increase in COX-2 expression in the up-regulation of HIF-1 α by PGE₂.

4. Discussion

In this study, we have shown that COX-2 activity is an absolute requirement for the pro-tumorigenic intracrine effects of PGE₂ in PC3 cells. Furthermore, our data show a novel positive feedback loop through which iPGE₂ up-regulates COX-2 and that might underlie the intracrine effects of PGE₂ in PC3 cells. Thus, the current data suggest that PGE₂ may contribute to the maintenance of prostate cancer cell growth by exerting an enhancing effect on its own synthesis through the continuous up-regulation of COX-2 by newly synthesized PGE₂.

PGE₂ has been previously found to induce COX-2 in mouse skin [28] and in several human cancer cell types including PC (both androgen-dependent and androgen-independent), breast and colon [18,19]. However, this is the first time, to the best of our knowledge, that it is shown that the pro-tumorigenic effects of PGE₂ are strictly dependent on COX-2 activity. Obviously further studies will be needed to determine whether COX-2 is equally relevant for the pro-tumorigenic effects of PGE₂ in cancer cell types other than PC3 cells. The same is true for the role of iPGE₂ in the up-regulation of COX-2, because our study has only demonstrated in PC3 cells the relevance of this intracrine mechanism of PGE₂ action. Regarding the implication of EP receptors in our current results, both transactivation of EGFR and EGFR-dependent increase in HIF-1 α expression in PGE₂-treated PC3 cells, are mediated by EP2 receptors [15].

We have previously shown that the stimulatory effect of PGE₂ on tumour phenotypes in PC3 cells (i.e. enhanced cell proliferation, migration, invasion, angiogenesis and loss of cell adhesion to collagen I) is critically dependent on the increase in HIF-1 α expression [15]. Here, we show that, in turn, COX-2 activity is required by PGE₂ for up-regulating HIF-1 α . Hence, we reasoned that the mechanism through which PGE₂ increases COX-2 expression in PC3 cells must be the essentially the same one that mediates the increase in HIF-1 α expression. Indeed, our data confirmed that, as for PGE₂-induced HIF-1 α up-regulation, PGE₂-induced COX-2 up-regulation involved the iPGE₂-dependent activation of EGFR through a Src-, Ca²⁺-dependent mechanism. Further studies demonstrated that the increasing effect of EGFR on COX-2 expression was mediated by kinases PI3K and MSK-1 (the latter previously activated by MAP kinases p38 and Erk1/2). Interestingly, most of the components of the pathway leading to COX-2 up-regulation, such as c-Src, EGFR, PI3k-Akt and Erk1/2, have been suggested to play relevant roles in prostate cancer, as indicated in several review articles [29-32]. Consequently, the positive feedback loop of COX-2 up-regulation triggered by iPGE₂ might also contribute to maintain a constant stimulation on these kinases involved in the progression of prostate cancer. Additional studies are needed to explore this hypothesis.

COX-2 is activated, at both the transcriptional and post-transcriptional levels, by growth factors, cytokines and mitogens [33]. COX-2-derived PGs stimulate cell proliferation, invasion, and angiogenesis and therefore COX-2 has been associated with malignant tumor growth and metastasis. Accordingly, COX-2 has been found at increased levels in PC as well as in other malignant neoplasms such as lung cancer, breast cancer and colon cancer [34]. COX-2 has been found upregulated throughout the entire prostate tumorigenic process (i.e. from hyperplasia to metastatic PC) [35] and it could contribute to the progression of PC since COX-2 expression is lower in T1-T2 stages than in T3-T4 stages [36]. Yet, the evidence from clinical studies on the protective effects of NSAIDs that preferentially inhibit COX-2 activity is far from being conclusive: while no special effect was found in several studies [37-41], in a couple of studies they reduced the risk of prostate cancer [34,42]. More recently, a pre-prostatectomy study on the biological effects of celecoxib or placebo found that celecoxib had no effect on apoptosis or androgen receptor levels in benign prostate or cancerous tissues [43]. Interestingly, in this study celecoxib did not reduce prostate prostaglandins [43], which might explain why celecoxib, and perhaps other anti-COX-2 agents, do not always demonstrate a beneficial effect in the prevention or treatment of prostate cancer. It should also be taken into account the possibility that overall

survival in studies reaching negative results might be enhanced if they had a longer duration. Whatever the reasons behind the absence of conclusive evidence on the protective effects on PC of NSAID that preferentially inhibit COX-2 activity, alternative PGE₂-based pharmacological approaches might be more effective considering that PGE₂ seems to be the main pro-carcinogenic prostanoid [6]. PGE₂-based pharmacological approaches might also have less safety concerns than COX-2 based ones -particularly unacceptable cardiovascular side effects- because they preserve production and effects of other prostanoids (for instance, anti-thrombotic prostacyclin) as they act downstream of COX-2 [6]. Since we have found that the pro-tumorigenic effects of PGE₂ in PC3 cells are critically dependent on its transport by PGT to the inside of the cell [14], it is relevant to evaluate whether inhibitors of PGT have better specificity for the prevention or treatment of PC and result in minimal adverse effects.

5. Conflicts of interest

There are no conflicts of interest to disclose.

Acknowledgments

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Legends to figures

Figure 1. PGE₂-induced increase in PC3 cell proliferation, migration, invasion and HIF-1 α expression, and PGE₂-induced decrease in PC3 cell adhesion to collagen I is prevented by COX inhibitors

a) *Cell proliferation (BrdU assay).* PC3 cells in serum free medium (24 h) were treated with COX inhibitors and then with PGE₂ for 1 h.

b) *Cell adhesion to collagen I.* Cell suspensions in serum free medium were treated COX inhibitors and then with PGE₂ for 30 min. PC3 cells were then plated on collagen I coated wells for 30 min.

c) *Cell migration.* The layer of confluent PC3 cells was wounded using sterile tips and treated with COX inhibitors and then with PGE₂.

d) *Cell invasion.* PC3 cells in suspension were treated with COX inhibitors, then with PGE₂ and seeded into transwell inserts coated with Matrigel basement membrane matrix. Cell invasion was assessed after 24 h.

e) *HIF-1 α expression.* Cells were treated with COX inhibitors and then with PGE₂ for 6 h. Normalized density ratio of HIF-1 α over β -actin is indicated for each band.

General information: Treatments: 1 h with 20 μ M diclofenac or 2 μ M celecoxib or 3 μ M nimesulide and then with 1 μ M PGE₂ (unless otherwise indicated). All experiments were repeated three times. Photographs are representative of the results obtained. Bars are mean \pm SD. Statistical analysis: * P < 0.01 vs other groups. ** P < 0.01 vs control and PGE₂.

Figure 2. PGE₂ up-regulates transcriptionally the expression of its own synthesizing enzyme COX-2 in a prostaglandin uptake transport (PGT)-dependent manner

a) *PGE₂ up-regulates COX-2.* PGE₂ up-regulates but not COX-1 (upper panel). Diclofenac-sensitive rise in the levels of iPGE₂ (Fig. 2 a middle panel). PGE₂ up-regulates transcriptionally COX-2 (lower panel). Left: Semi-quantitative RT-PCR. Right: PC3 cells were treated for 1 h with 1 μ g/ml actinomycin D (Act. D) and then with PGE₂ for 2 h.

b) *PGE₂ up-regulates COX-2 in a PGT-dependent manner.* Upper panel: Internalization of PGE₂ in a PGT inhibitor-sensitive manner. Cells were pre-incubated for 1 h with the inhibitor of PGT bromosulphophthalein (Bs, 10 μM) and then with PGE₂ for 2 min. Afterwards, cells were fixed, permeabilized and subjected to immunofluorescence analysis after incubation with anti-PGE₂ antibody. Images were analysed using ImageJ software (NIH). Middle panel, left: siRNA PGT prevents PGE₂-induced COX-2 up-regulation. Transfected cells were treated with PGE₂ for 2 h. Inset: siRNA PGT blunts the constitutive expression of PGT. Middle panel, right: Bs prevents PGE₂-induced COX-2 up-regulation. Treatments: 10 μM Bs/1 h and then PGE₂/2 h. Lower panel: Bs prevents PGE₂-induced increase in the activity of a human COX-2 gene promoter. Treatments: 10 μM Bs/1h and then PGE₂/30 min. Each bar represents the mean ± SD of the luciferase activity (relative luminescence units (RLU)) normalized by Renilla luciferase activity. Statistical analysis: * P < 0.01 vs other groups.

General information: PC3 cells were treated with 1 μM PGE₂. Western blot analysis: Normalized density ratio of COX-1 or COX-2 over β-actin is indicated for each band. RT-PCR: Equal mRNA loading was confirmed by assessing the expression of GAPDH mRNA. Normalized density ratio of COX-2 over GAPDH is indicated for each band. All experiments were repeated three times.

Figure 3. c-Src-dependent activation of EGFR mediates the up-regulation of COX-2 by PGE₂.

a) *The inhibitor of EGFR activation AG1478 prevents the up-regulation of COX-2 by PGE₂.* Upper panel Treatments: 1 μM AG1478/1h and then PGE₂ for 2 h (left) or 20 min (right). Lower panel: Transfected cells were treated with AG1478 and then with PGE₂ for 30 min. Luciferase activity was expressed as in Figure 2 b. Statistical analysis: * P < 0.01 vs

b) *Treatment with PGE₂ results in Bs-sensitive increase in EGFR tyrosine phosphorylation.* Serum starved cells were treated with 10 μM Bs/1h and then with PGE₂/30 s. Inset: PGE₂ increases EGFR tyrosine phosphorylation.

c) *PGE₂-induced tyrosin phosphorylation of EGFR is dependent on c-Src.* Serum starved cells were treated with the inhibitor of c-Src phosphorylation PP2 (10 μM/1 h) and then with PGE₂ for 30 s. Inset: PGE₂ increases c-Src phosphorylation.

d) *The inhibitor of c-Src phosphorylation PP2 prevents the up-regulation of COX-2 by PGE₂.* Serum starved cells were treated with the inhibitor of c-Src phosphorylation PP2 (10 μM/1 h) and then with PGE₂ for 2 h

General information: All experiments were repeated three times. PC3 cells were treated with 1 μM PGE₂. Western blot analysis: Normalized density ratio of COX-1, COX-2, p-EGFR and p-Src over β-actin is indicated for each band. Autoradiographs are representative examples of at least three independent experiments. RT-PCR: Equal mRNA loading was confirmed by assessing the expression of GAPDH mRNA. Normalized density ratio of COX-2 over GAPDH is indicated for each band.

Figure 4. Erk1/2/p38/MSK-1 and PI3k/Akt pathways mediate the EGFR-dependent-increase in COX-2 expression in PC3 cells upon treatment with PGE₂

a) *Inhibition of Erk1/2/p38/MSK-1 and PI3k/Akt pathways prevents the up-regulation of COX-2 by PGE₂ PC3 cells.* Upper panel PC3 cells were pre-incubated with pharmacological inhibitors of both pathways (MSK-1 was also knocked down as shown in the right panel) before being treated with PGE₂ for 2 h. Lower panel: Cells which were transfected with a human COX-2 gene promoter were pretreated with the kinase inhibitors and treated with PGE₂ for 30 min. Luciferase activity was expressed as in Fig. 2b. Statistical analysis: * P < 0.01 vs other groups.

b) *PGE₂ increases Erk1/2, p38, MSK-1 and Akt phosphorylation.*

c) *PGE₂ increases the phosphorylation of Erk1/2, p38, MSK-1 and Akt in an EGFR-dependent manner.* Cells were treated for 1 h with AG1478 and then with PGE₂ for 5 min (p- Erk1/2, p-p38 and p-MSK-1) or 10 min (p-Akt).

d) *PGE₂-induced increase in MSK1 phosphorylation is sensitive to inhibition of Erk1/2 and p38 but not to inhibition of PI3K.* Cells were treated with the kinase inhibitors before being treated with PGE₂ for 5 min.

General information: Treatments: PC3 cells were treated for 1 h with 20 μM PD98059, 10 μM SB203580, 1 μM H89, 10 μM LY294002, 1 μM wortmannin or 1 μM AG1478 and then cells were treated with 1 μM PGE₂. Western blot autoradiographs are representative examples of at least three independent experiments. Normalized density ratio of COX-2, p-Erk1/2, p-p38, p-MSK-1 and p-Akt and over β-actin is indicated for each band.

Figure 5. PGE₂ increases transcriptionally the expression of HIF-1α in PC3 cells through an intracrine mechanism involving the same signaling pathways that mediate COX-2 up-regulation

a) *PGE₂ increases transcriptionally the expression of HIF-1α.* Left: PGE₂ increases the expression of HIF-1α mRNA Right: Actinomycin D (Act. D) prevents PGE₂-induced HIF-1α up-regulation. PC3 cells were treated with actinomycin D (Act. D) and then with PGE₂ for 2 h. Inset: PGE₂ does not increase the stability of HIF-1α Cells were incubated for 6 h with or without PGE₂. Thereafter, the protein translation inhibitor cycloheximide (CHX, 50 μg/ml) was added for up to 30 min.

b) *PGE₂ increases the expression of HIF-1α in PC3 cells through a Src, EGFR, Erk1/2, p38, MSK-1 and PI3K-dependent intracrine mechanism.* Upper panel Left: siRNA PGT prevents PGE₂-induced HIF-1α up-regulation. Right: The inhibitor of c-Src phosphorylation PP2 prevents the up-regulation of HIF-1α by PGE₂. Middle panel Left: The inhibitor of EGFR activation AG1478 prevents the up-regulation of HIF-1α by PGE₂. Right: PGE₂-induced increase in HIF-1α expression is sensitive to inhibitors of Erk1/2 and p38. Lower panel Left: siRNA MSK-1 prevents PGE₂-induced HIF-1α up-regulation. Inset: The inhibitor of MSK1 H89 prevents the up-regulation of HIF-1α by PGE₂. Right: Inhibitors of PI3K activation prevent the up-regulation of HIF-1α by PGE₂.

General information: Pretreatments: 1 μg/ml Act. D, 10 μM PP2, 1 μM AG1478, 20 μM PD98059, 10 μM SB203580, 1 μM H89, 10 μM LY294002 or 1 μM wortmannin. All pretreatments lasted 1 h and then cells were treated with 1 μM PGE₂ for 6 h (unless otherwise indicated). Western blot autoradiographs are representative examples of at least three independent experiments. Normalized density ratio of HIF-1α over β-actin is indicated for each band.

Figure 6. Proposed pathway by which PGE₂ up-regulates COX-2

Legends to supplementary figures

General information: These figures show the quantification of the experiments involving Western blot analysis or semiquantitative RT-PCR. Data are mean SD (fold change over control) of the densitometric analysis of three different experiments in which protein expression or mRNA expressions normalized to β-actin. Treatments: PC3 cells were treated for 1 h with 20 μM diclofenac, 2 μM celecoxib, 3 μM nimesulide, 1 μg/ml actinomycin D (Act. D), 10 μM bromosulphophthalein (Bs), 10 μM PP2, 1 μM AG1478, 20 μM PD98059, 10 μM SB203580, 1 μM H89, 10 μM LY294002, 1 μM wortmannin or 1 μM AG1478 Unless otherwise indicated, 1 μM PGE₂ was used in the experiments. a.u.: arbitrary units.

Figure S.1. PGE₂-induced increase in HIF-1 α expression is prevented by COX inhibitors (quantification of the experiments shown in Fig. 1e). Cells were treated with PGE₂ for 6 h *P < 0.05 vs other groups.

Figure S.2. PGE₂ up-regulates transcriptionally the expression of its own synthesizing enzyme COX-2 in a prostaglandin uptake transport (PGT)-dependent manner

a) PGE₂ up-regulates COX-2. PGE₂ up-regulates COX-2 but not COX-1 (upper panel) **P < 0.05 vs control PGE₂ up-regulates transcriptionally COX-2 (lower panel). Left: Semi-quantitative RT-PCR. Right: PC3 cells were treated for 1 h with Act. D and then with PGE₂ for 2 h. **P < 0.05 vs control *P < 0.05 vs other groups.

b) PGE₂ up-regulates COX-2 in a PGT-dependent manner: siRNA PGT prevents PGE₂-induced COX-2 up-regulation Transfected cells were treated with PGE₂ for 2 h. *P < 0.05 vs other groups. Inset: siRNA PGT blunts the constitutive expression of PGT. *P < 0.05 vs other groups. Right: Bromosulphophthalein (Bs) prevents PGE₂-induced COX-2 up-regulation. Treatments: Bs/1 h and then PGE₂/2 h. *P < 0.05 vs other groups.

Figure S.3. c-Src-dependent activation of EGFR mediates the up-regulation of COX-2 by PGE₂.

a) The inhibitor of EGFR activation AG1478 prevents the up-regulation of COX-2 by PGE₂. Treatments: 1 μ M AG1478/1h and then PGE₂ for 2 h (left) or 20 min (right). *P < 0.05 vs other groups.

b) Treatment with PGE₂ results in Bs-sensitive increase in EGFR tyrosine phosphorylation. Serum starved cells were treated with 10 μ M Bs/1h and then with PGE₂/30 s. *P < 0.05 vs other groups. Inset: PGE₂ increases EGFR tyrosine phosphorylation. *P < 0.05 vs other groups.

c) PGE₂-induced tyrosin phosphorylation of EGFR is dependent on c-Src. Serum starved cells were treated with the inhibitor of c-Src phosphorylation PP2 and then with PGE₂ for 30 s. *P < 0.05 vs other groups. Inset: PGE₂ increases c-Src phosphorylation. *P < 0.05 vs other groups.

d) The inhibitor of c-Src phosphorylation PP2 prevents the up-regulation of COX-2 by PGE₂. Serum starved cells were treated with the inhibitor of c-Src phosphorylation PP2 and then with PGE₂ for 2 h *P < 0.05 vs other groups.

Figure S.4. Erk1/2/p38/MSK-1 and PI3k/Akt pathways mediate the EGFR-dependent-increase in COX-2 expression in PC3 cells upon treatment with PGE₂

a) Inhibition of Erk1/2/p38/MSK-1 and PI3k/Akt pathways prevents the up-regulation of COX-2 by PGE₂ PC3 cells. Cells were pre-incubated with pharmacological inhibitors of both pathways (except for MSK-1, in which cells were transfected with siRNA MSK-1) before being treated with PGE₂ for 2 h. * P < 0.05 vs other groups.

b) PGE₂ increases Erk1/2, p38, MSK-1 and Akt phosphorylation. * P < 0.05 vs other groups.

c) PGE₂ increases the phosphorylation of Erk1/2, p38, MSK-1 and Akt in an EGFR-dependent manner. Cells were treated for 1 h with AG1478 and then with PGE₂ for 5 min (p- Erk1/2, p-p38 and p-MSK-1) or 10 min (p-Akt). * P < 0.05 vs other groups. ** P < 0.05 vs control and PGE₂.

d) PGE₂-induced increase in MSK1 phosphorylation is sensitive to inhibition of Erk1/2 and p38 but not to inhibition of PI3K. Cells were treated with the kinase inhibitors before being treated with PGE₂ for 5 min. * P < 0.05 vs other groups.

Figure S.5. PGE₂ increases transcriptionally the expression of HIF-1 α in PC3 cells through an intracrine mechanism involving the same signaling pathways that mediate COX-2 up-regulation

a) PGE₂ increases transcriptionally the expression of HIF-1 α . Left: PGE₂ increases the expression of HIF-1 α mRNA **P < 0.05 vs control. Right: Actinomycin D (Act. D) prevents PGE₂-induced HIF-1 α up-regulation. PC3 cells were treated with actinomycin D (Act. D) and then with PGE₂ for 2 h. * P < 0.05 vs other groups. Inset: PGE₂ does not increase the stability of HIF-1 α Cells were incubated for 6 h with or without PGE₂. Thereafter, the protein translation inhibitor cycloheximide (CHX, 50 μ g/ml) was added for up to 30 min. No statistically significant differences were found between control and PGE₂.

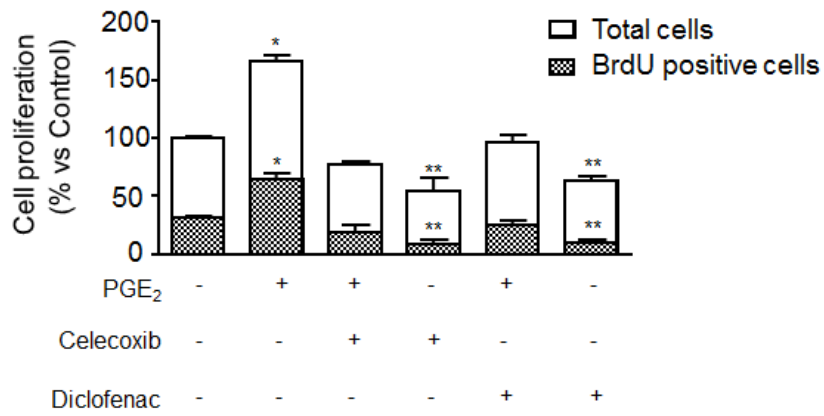
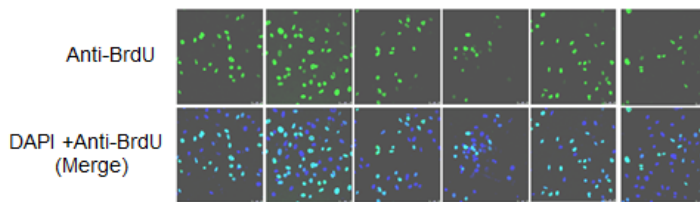
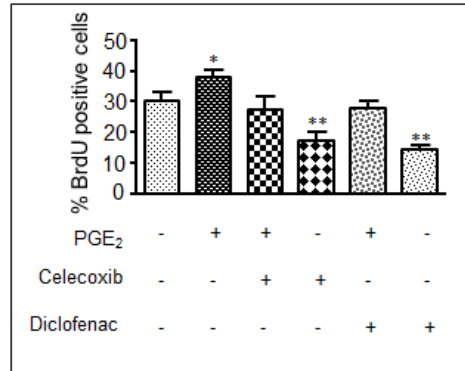
b) PGE₂ increases the expression of HIF-1 α in PC3 cells through a Src, EGFR, Erk1/2, p38, MSK-1 and PI3K-dependent intracrine mechanism. Upper panel Left: siRNA PGT prevents PGE₂-induced HIF-1 α up-regulation. *P < 0.05 vs other groups. **P < 0.05 vs control and PGE₂. Right: The inhibitor of c-Src phosphorylation PP2 prevents the up-regulation of HIF-1 α by PGE₂ *P < 0.05 vs other groups. **P < 0.05 vs control and PGE₂. Middle panel Left: The inhibitor of EGFR activation AG1478 prevents the up-regulation of HIF-1 α by PGE₂. *P < 0.05 vs other groups. Right: PGE₂-induced increase in HIF-1 α expression is sensitive to inhibitors of Erk1/2 and p38. *P < 0.05 vs other groups. Lower panel Left: The inhibitor of MSK1 H89 prevents the up-regulation of HIF-1 α by PGE₂. *P < 0.05 vs other groups. Right: Inhibitors of PI3K activation prevent the up-regulation of HIF-1 α by PGE₂. *P < 0.05 vs other groups.

Figure S.6. Effect of the inhibitors used in this study in the basal expression of COX-2

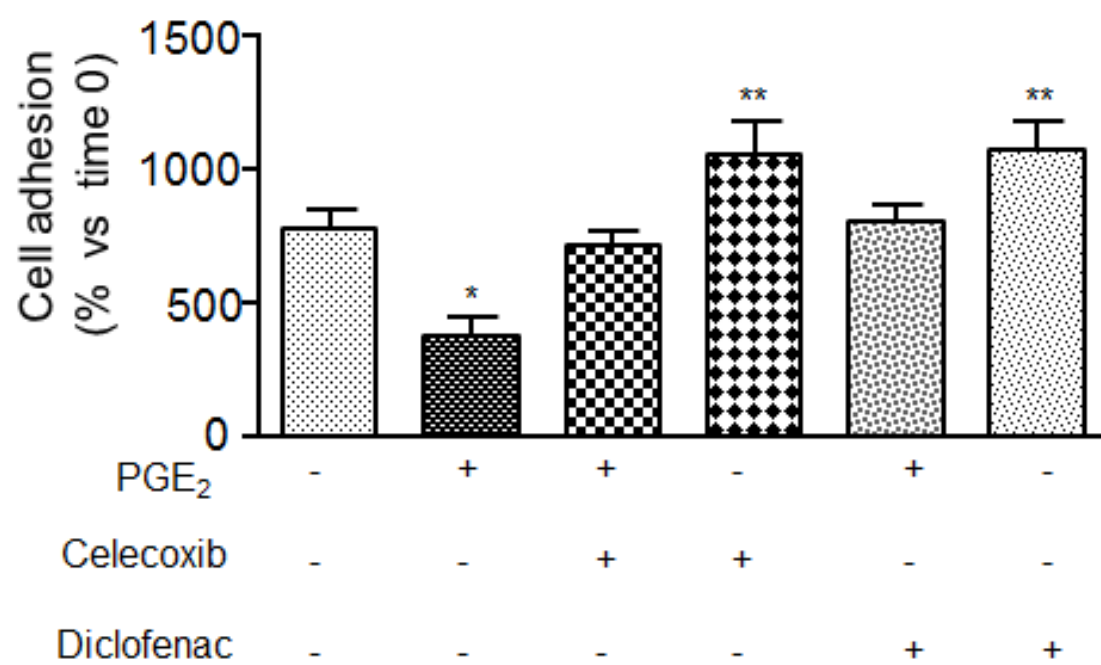
Cells were treated for 7 h with the inhibitors *P < 0.05 vs other groups.

Fig1

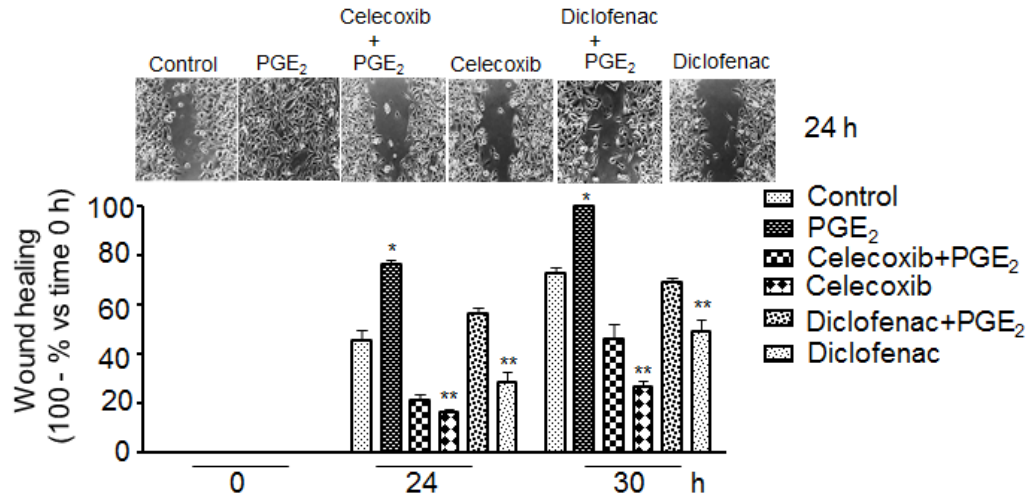
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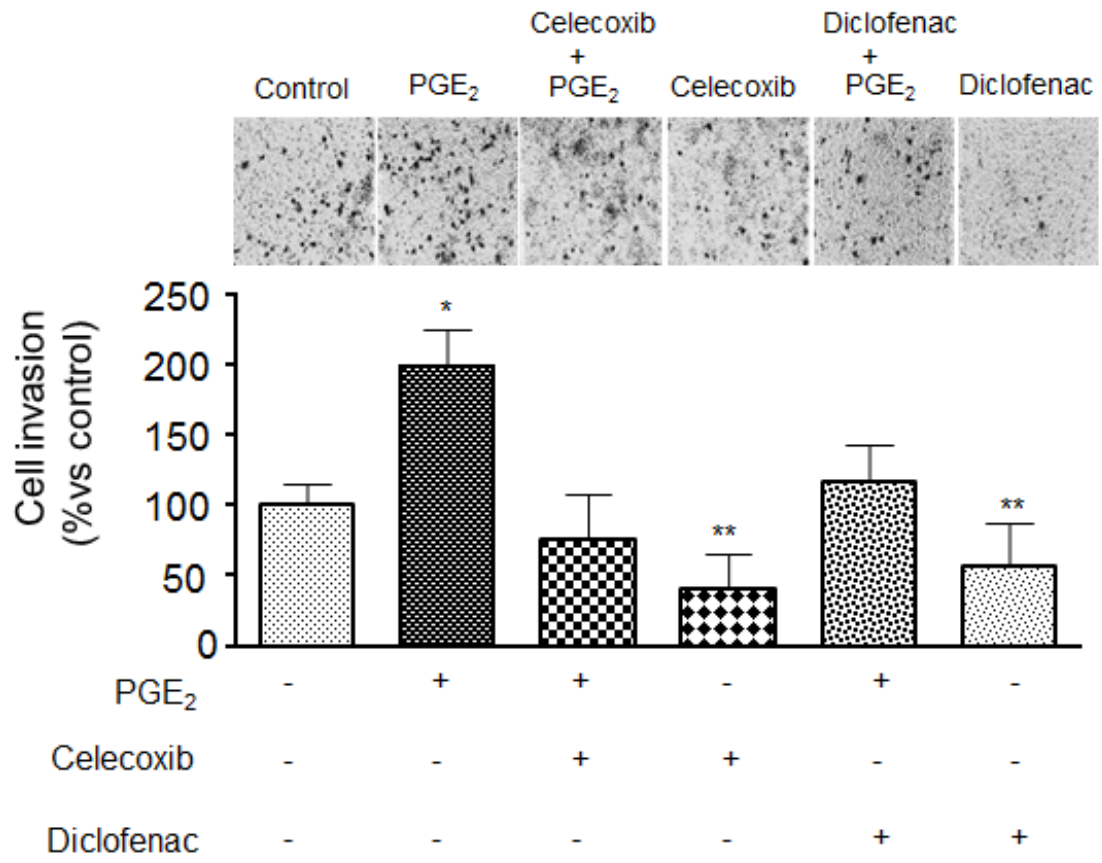
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c)



d)



e)

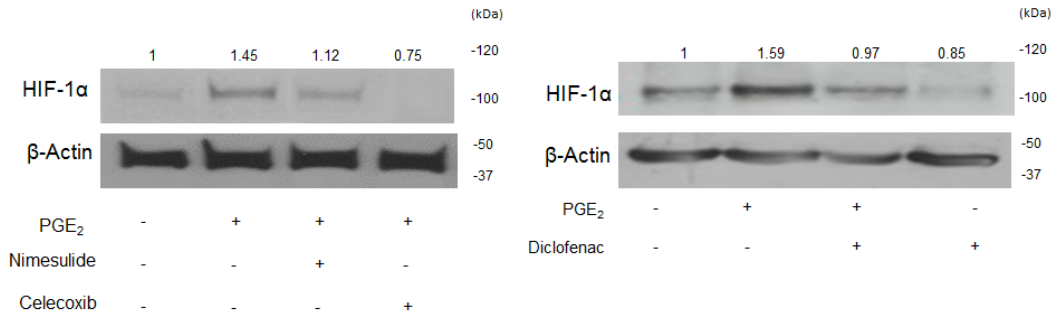
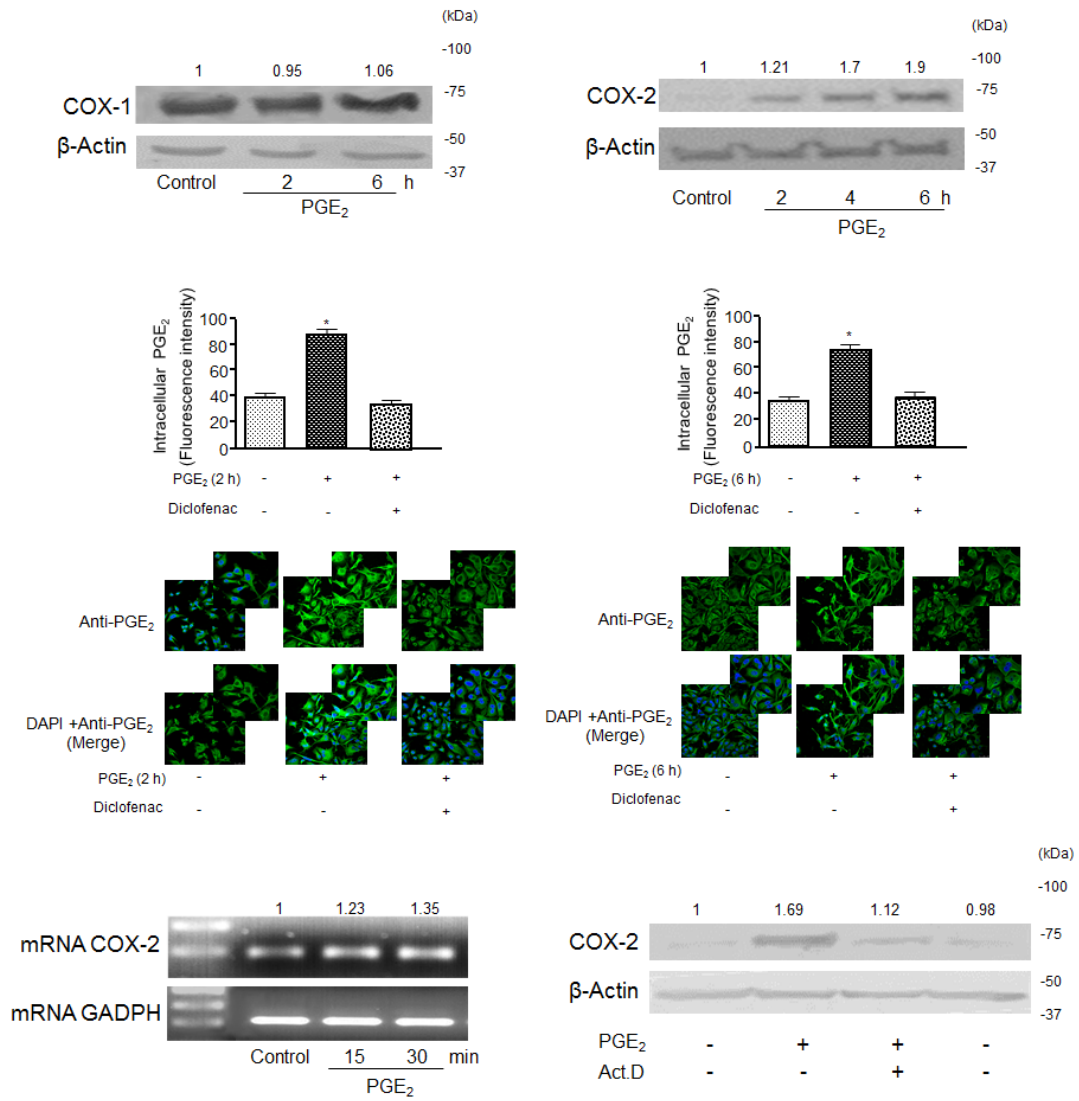


Fig2

a)



b)

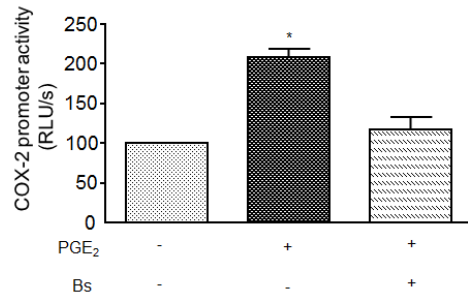
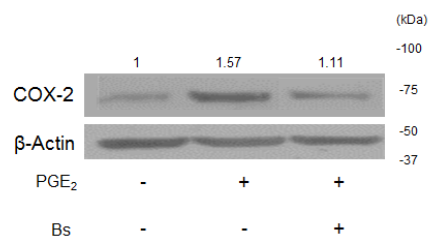
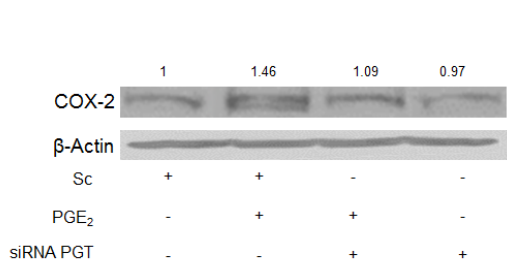
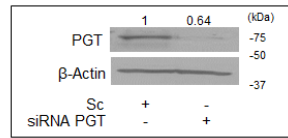
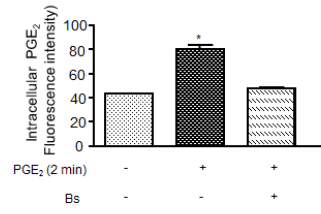
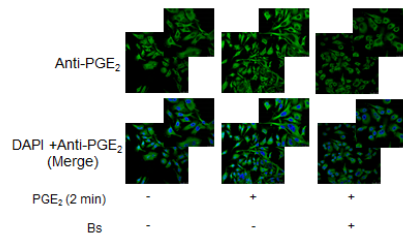
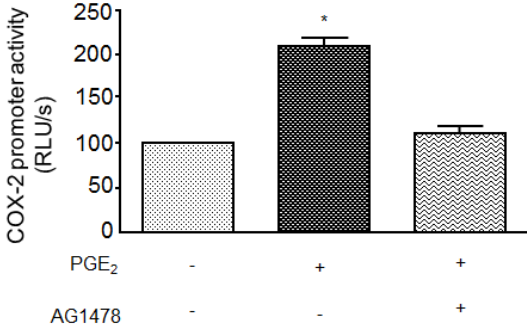
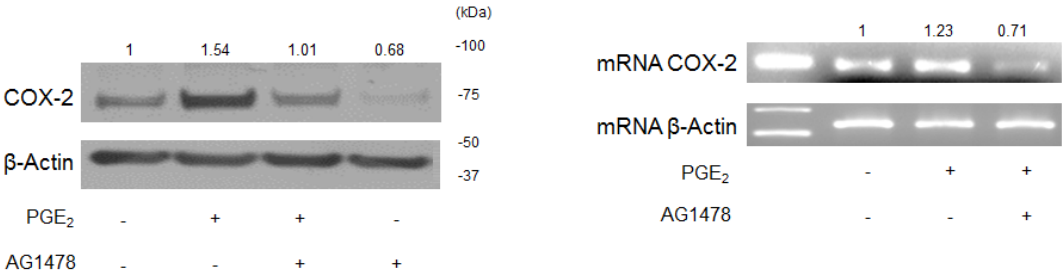
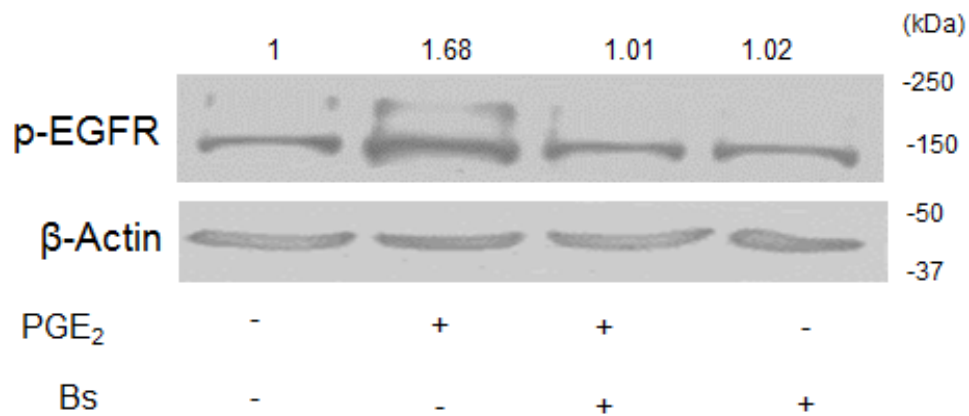
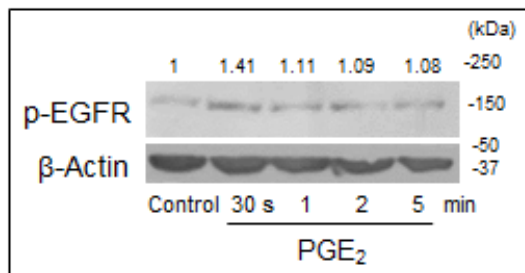


Fig3

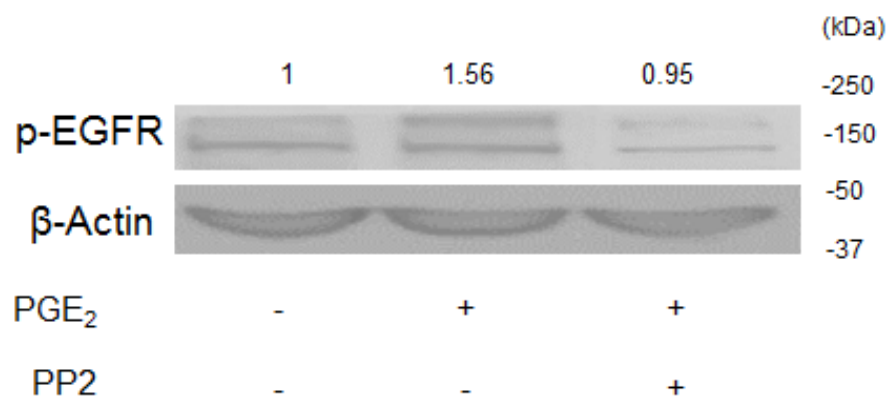
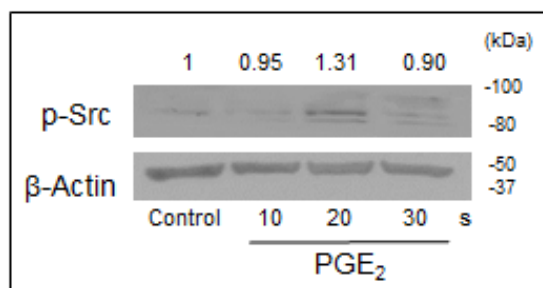
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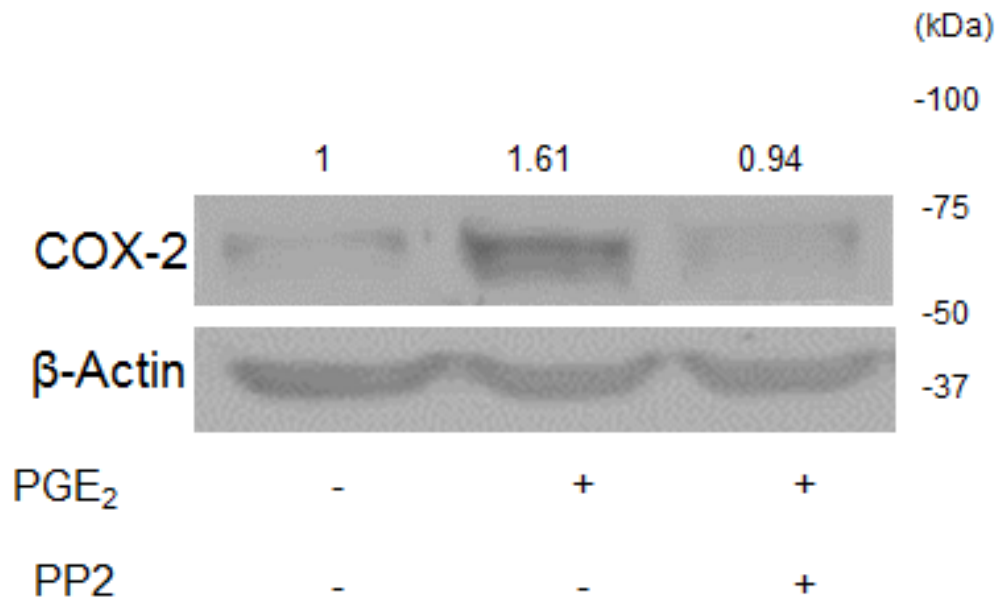
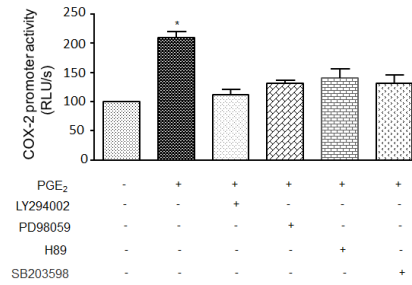
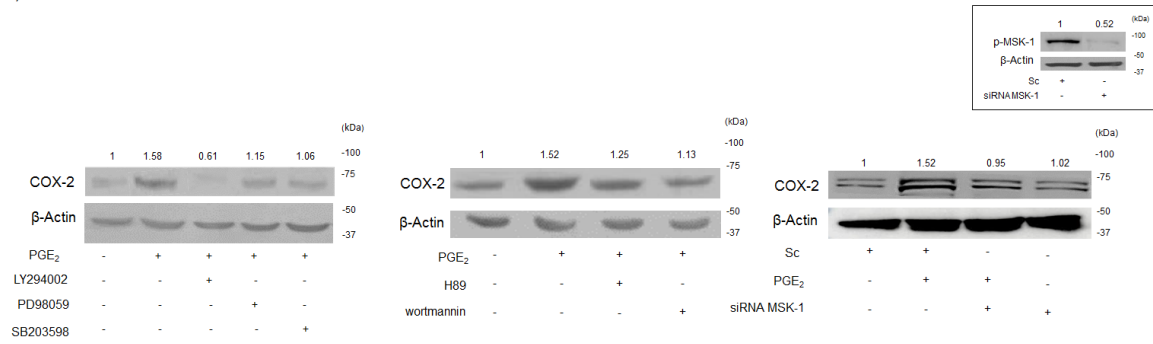
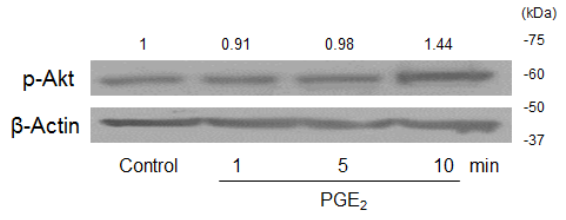
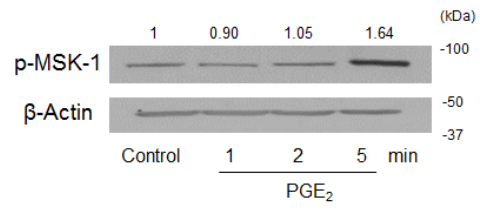
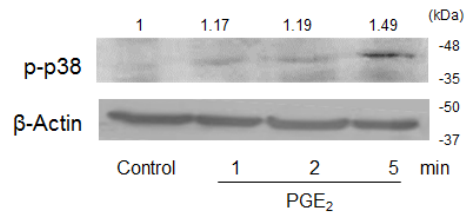
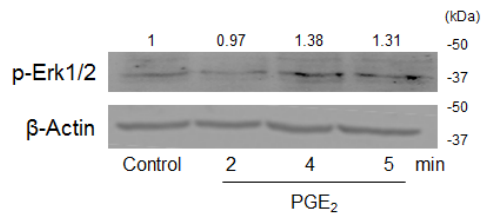


Fig4

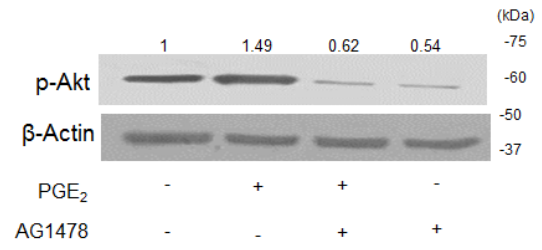
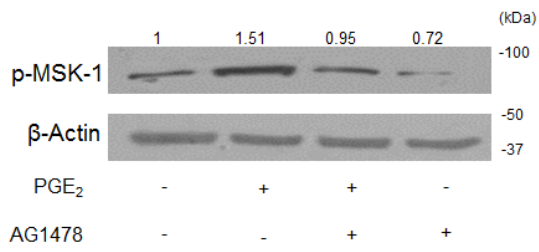
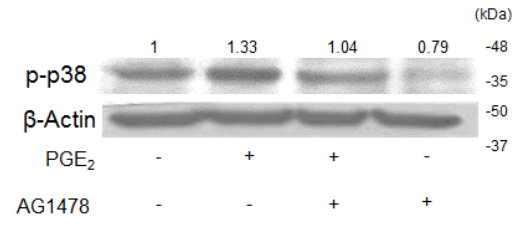
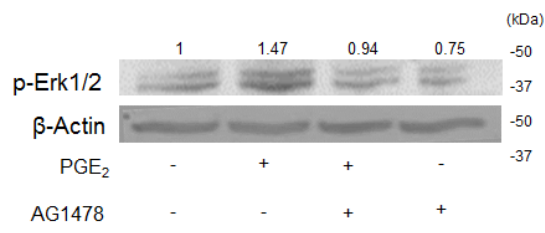
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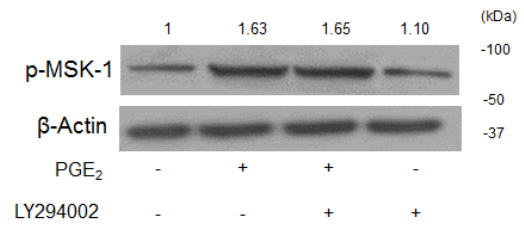
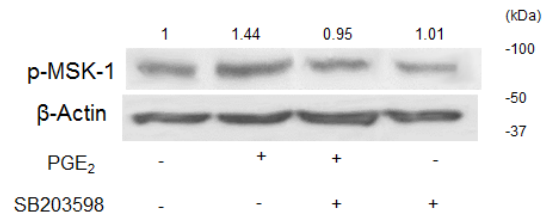
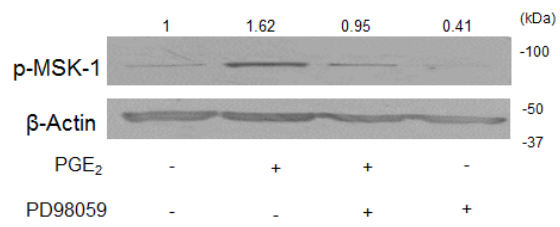
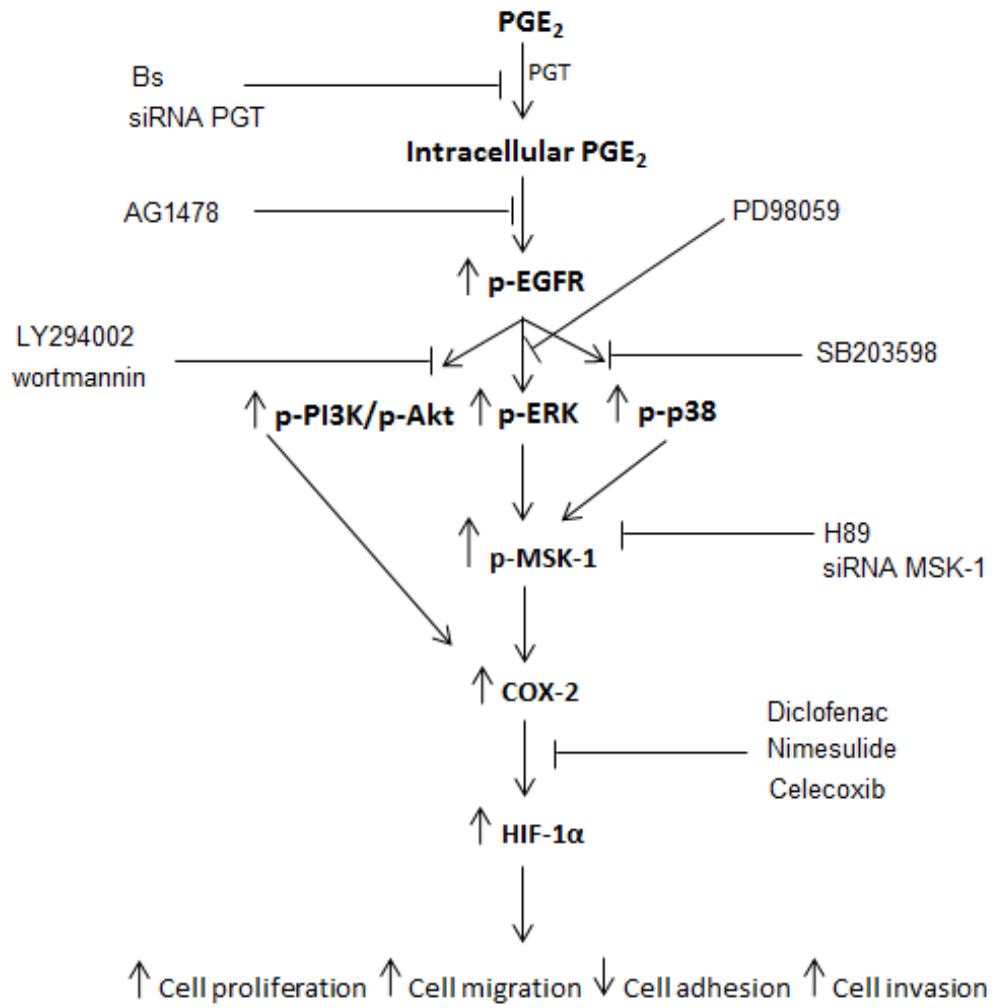
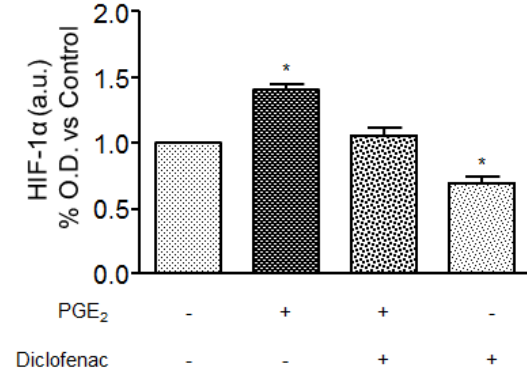
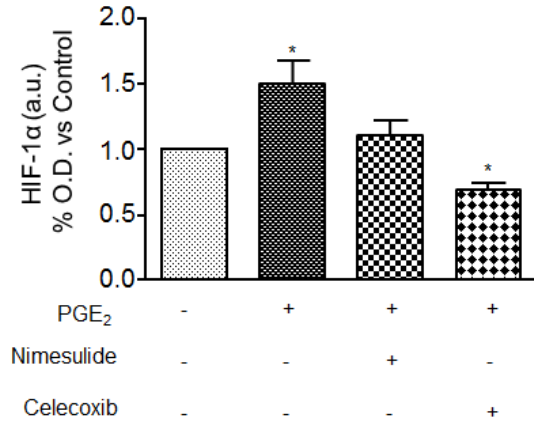


Fig6



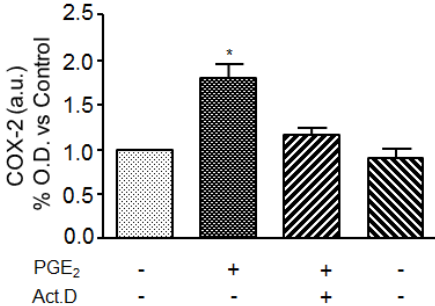
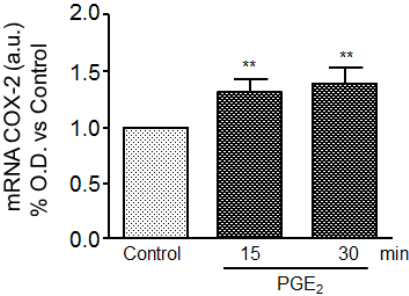
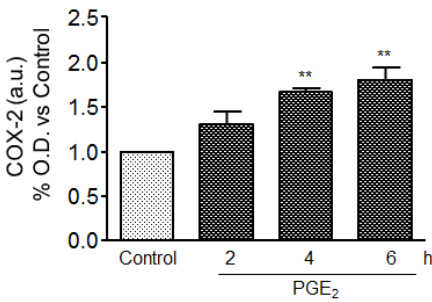
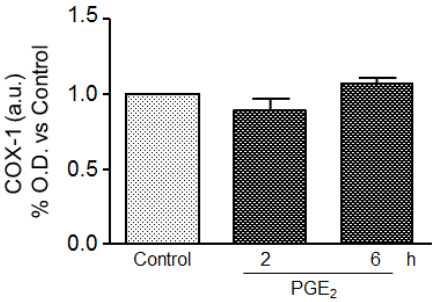
FigS1

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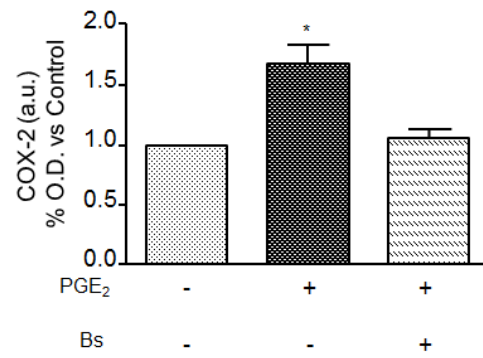
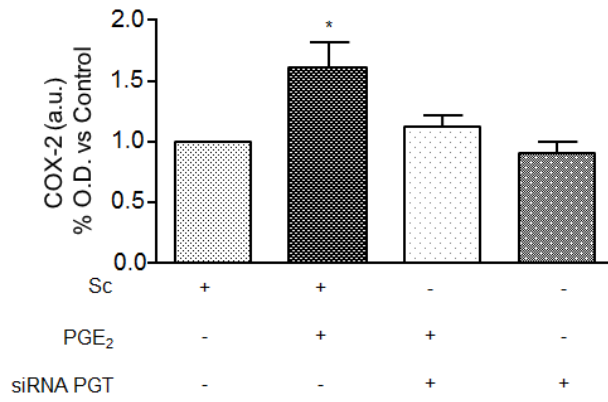
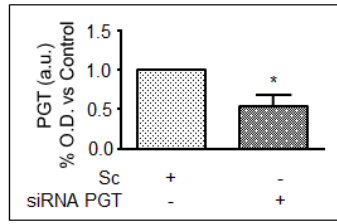


FigS2

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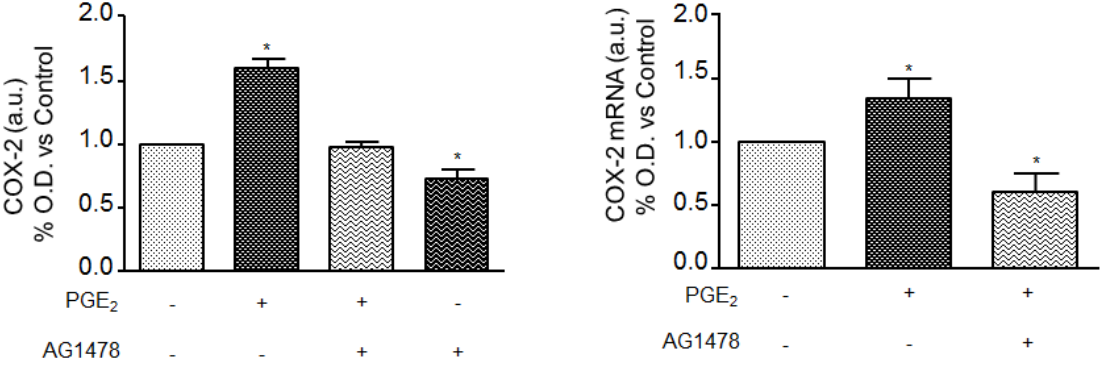


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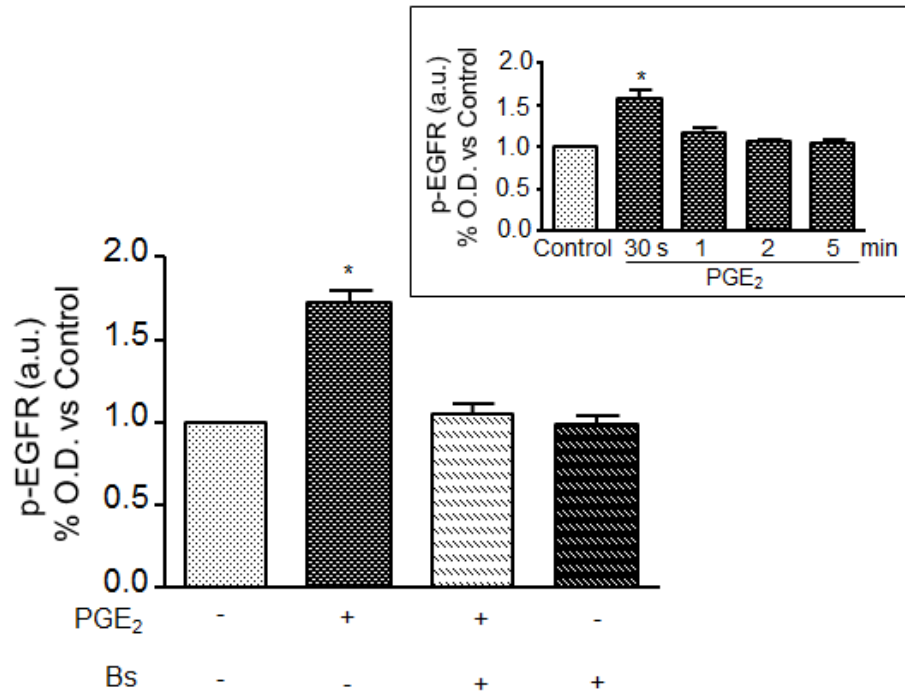


FigS3

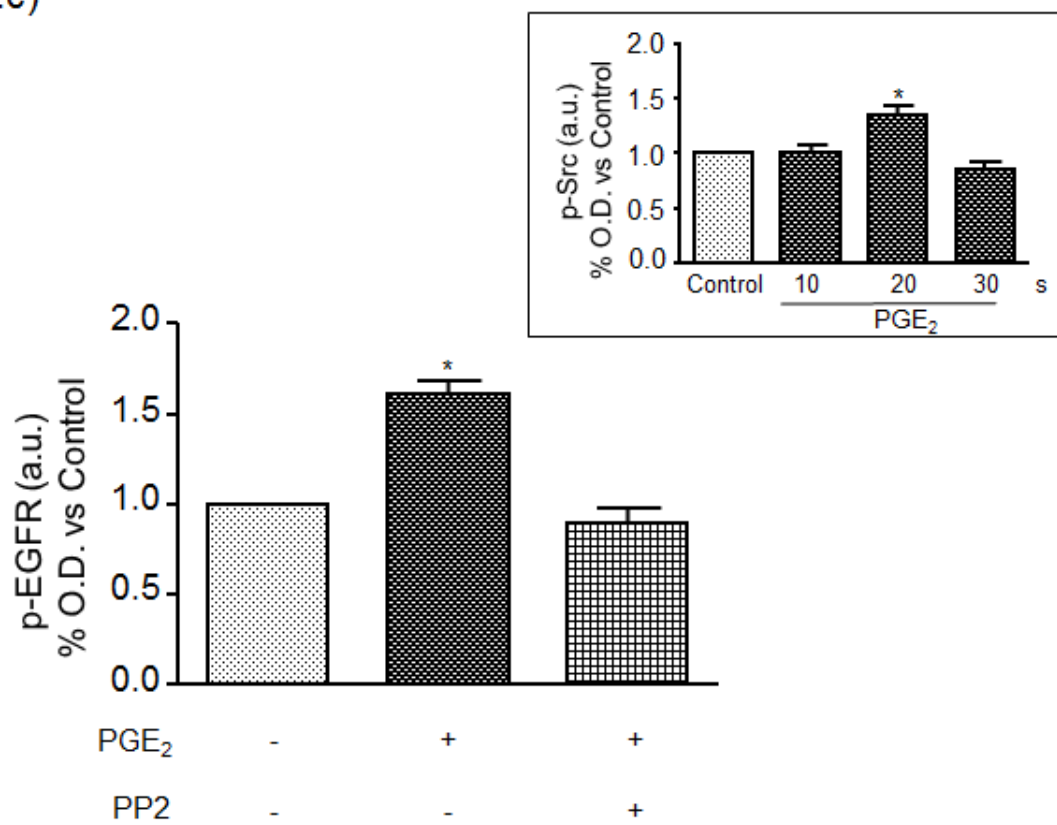
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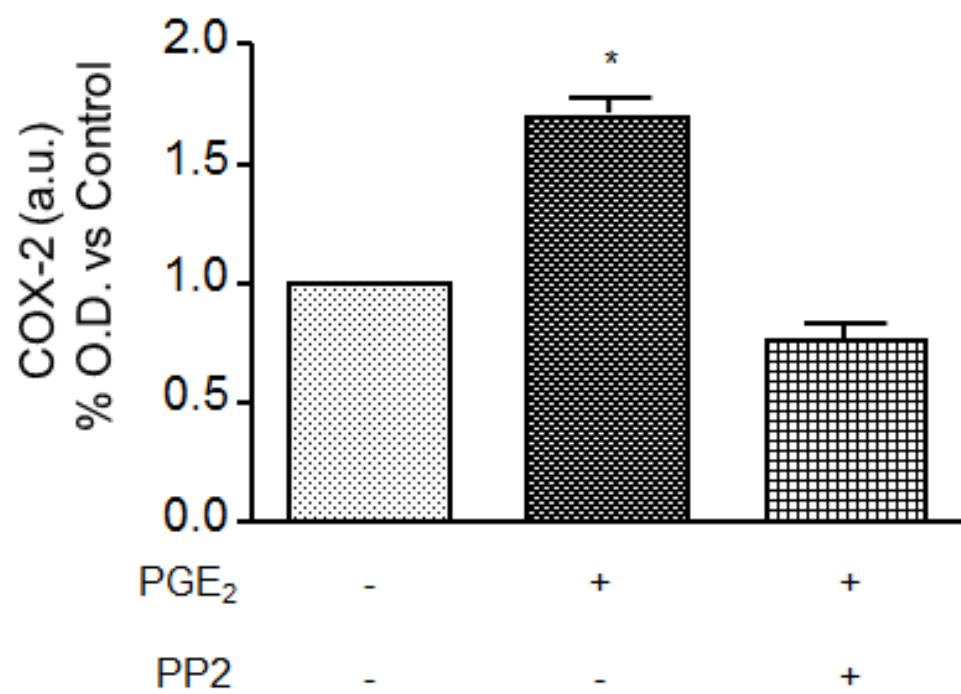
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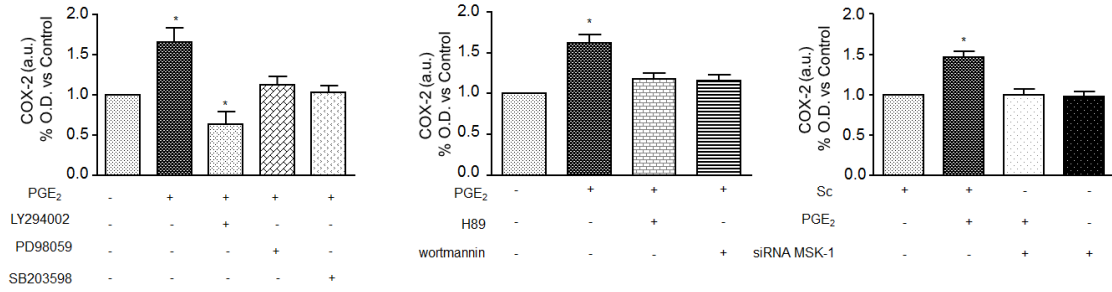


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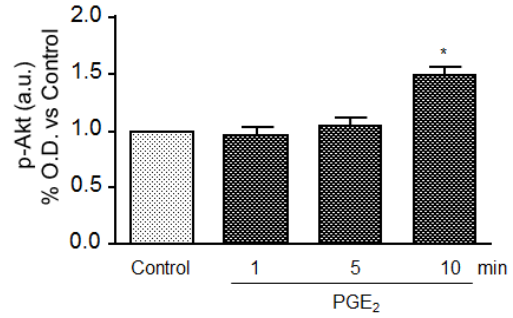
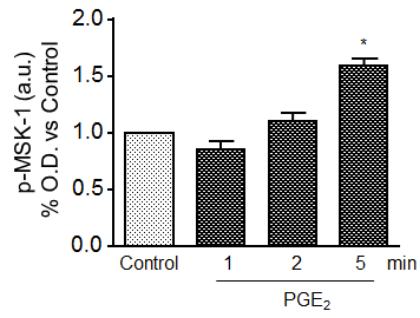
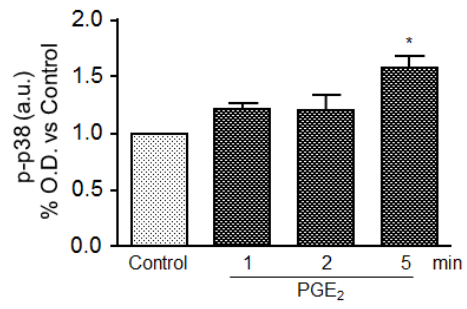
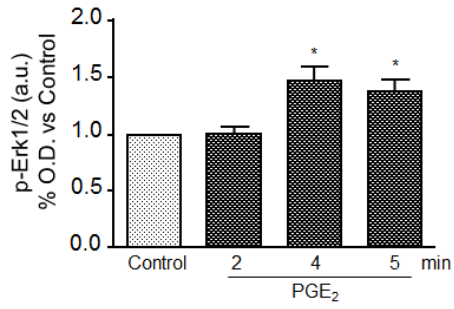


FigS4

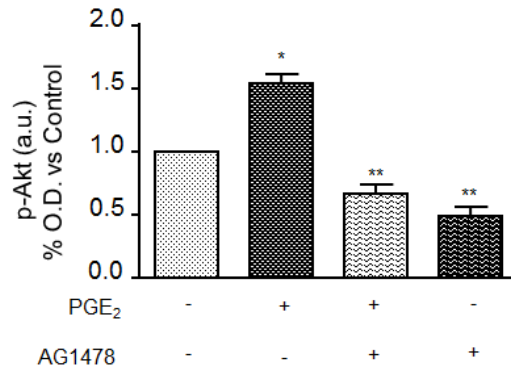
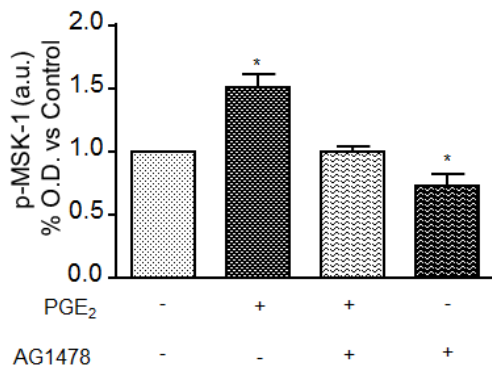
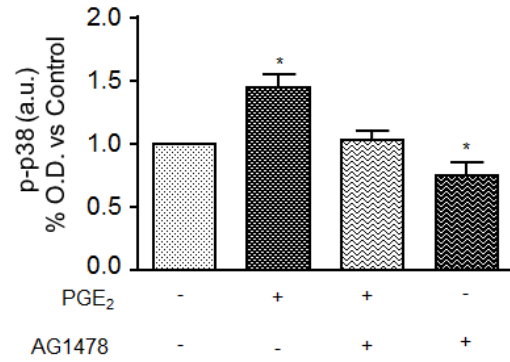
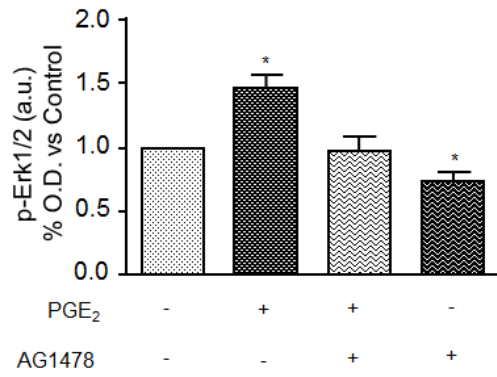
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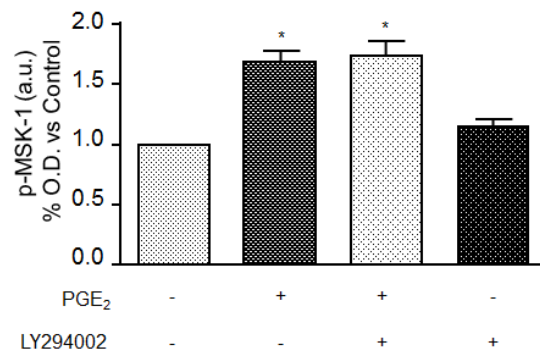
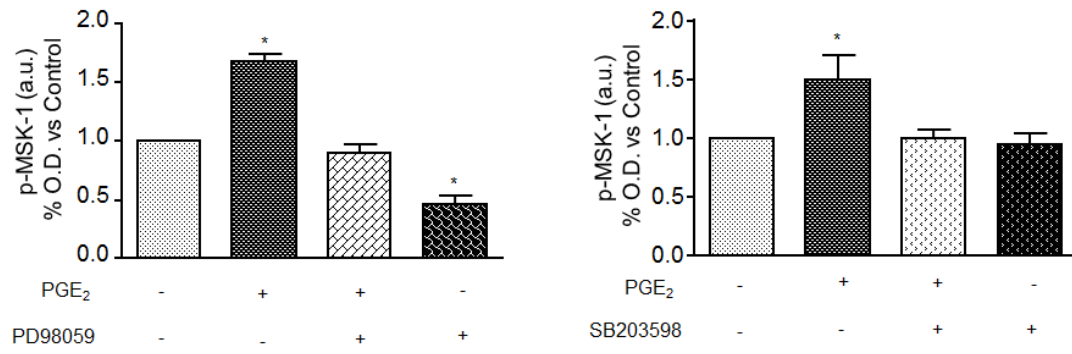
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S.4.c)

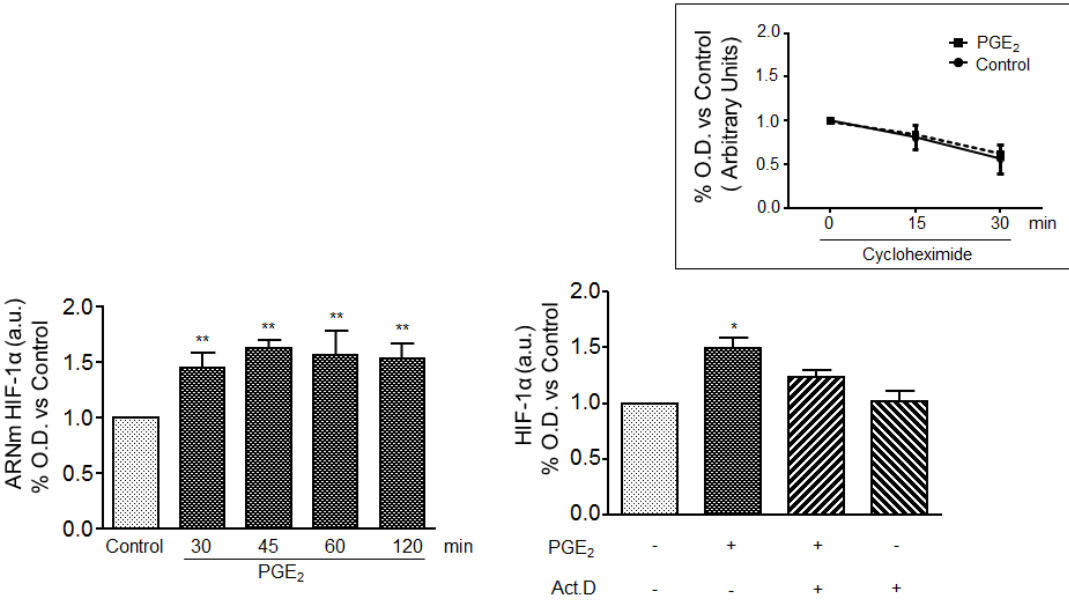


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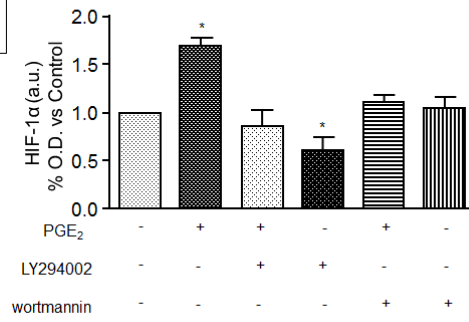
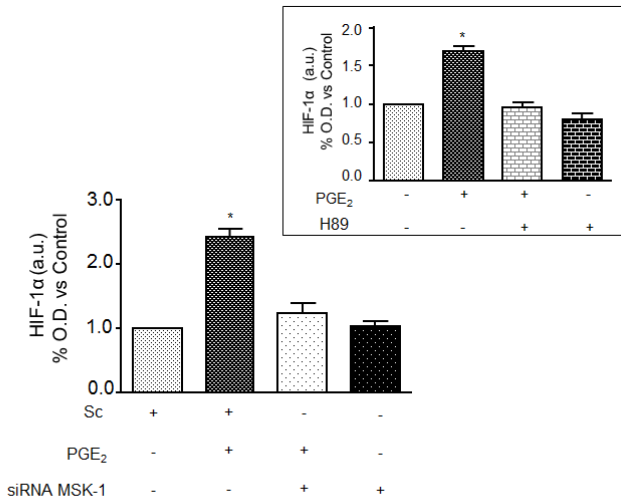
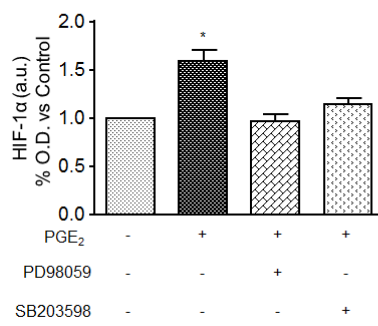
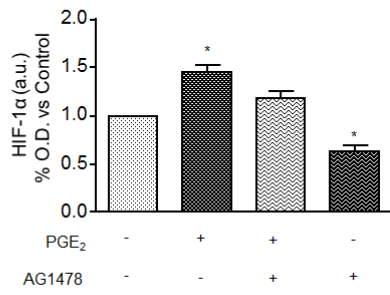
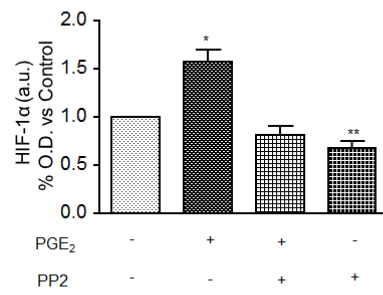
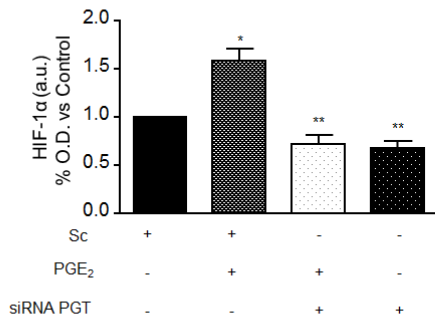


FigS5

S.5.a)



S.5.b)



FigS6

S.6)

