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# Prostaglandin transporter PGT as a new pharmacological target in the prevention of inflammatory cytokine-induced injury in renal proximal tubular HK-2 cells

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SUMMARY

*Aims:* Inflammatory cytokines contribute to proximal tubular cell (PTC) injury leading to the deterioration of renal function and acute kidney injury (AKI) development. They also stimulate cyclo-oxygenase-2 (COX-2)-dependent production and release to the extracellular medium of prostaglandin $E_2$ ($PGE_2$), a mediator of PTC injury. However, in several settings $PGE_2$ re-uptake by prostaglandin transporter (PGT) is critical for $PGE_2$-mediated PTC injury. Here we investigated several deleterious effects of pro-inflammatory cytokines in PTC and their prevention by PGT targeting.

*Main methods:* In human kidney-2 (HK-2) PTC exposed to an inflammatory cytokine cocktail, consisting of interleukins (IL) IL-1α, IL-1β and IL-2, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), were determined the changes in several parameters related to PTC injury, their dependency on $PGE_2$ (through modulation by antagonists of $PGE_2$ receptors) and the preventive effect of PGT inhibitor bromosulfophthalein.

*Key findings:* The cytokine cocktail induced a COX-2-dependent increase in intracellular $PGE_2$ ($iPGE_2$) and cell death, together to a decrease in cell number and cell proliferation. There was also loss of adherent cells to collagen IV, changes in actin cytoskeleton and loss of monolayer integrity, together to an increase in paracellular permeability. All the changes were sensitive to antagonist of $PGE_2$ receptors AH6809 and were fully prevented by bromosulfophthalein.

*Significance:* These results indicate that PGT-, $iPGE_2$-dependent mechanisms mediate inflammatory cytokine-induced HK-2 cell injury and suggest that treatment with PGT inhibitors might help to prevent AKI induced by sepsis, renal ischemia/reperfusion and other pathological conditions in which inflammatory cytokines contribute to PTC damage.

## 1. Introduction

Inflammation participates in the pathogenesis of renal disease and this is particularly true for acute kidney disease (AKI), which associates with intrarenal and systemic inflammation [1]. Thus, inflammation has been involved in the pathogenesis of AKI induced by ischemia-reperfusion injury (which may be superimposed on diabetes, hypertension and/or chronic kidney disease), nephrotoxins such as cisplatin or gentamicin (among many others) or sepsis [1]. Epithelial cells of the proximal tubule are mostly affected in the main causes of AKI and the enhanced release of inflammatory mediators has a pivotal role in proximal tubular cell (PTC) injury leading to the deterioration of renal

*Abbreviations:* AKI, acute kidney injury; COX-2, ciclo-oxygenase 2; EP, prostaglandin $E_2$ receptors; HK-2, human kidney 2; IL, interleukin; IFN-γ, interferon γ; $iPGE_2$, intracellular prostaglandin $E_2$; $PGE_2$, prostaglandin $E_2$; PGT, prostaglandin transporter; PTC, proximal tubular cells; TNF-α, tumour necrosis factor α.

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function and AKI development [2]. Several cytokines such as interleukins IL-1α, IL-1β and IL-2, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are inflammatory mediators able to cause PTC death in vitro [3–5] and therefore they might contribute to the genesis of AKI. These pro-inflammatory cytokines are also able to increase the expression of cyclo-oxygenase-2 (COX-2) [6], an enzyme involved in the regulation of inflammatory processes through its products, mainly prostaglandin $E_2$ ($PGE_2$) [7]. Interestingly, $PGE_2$ plays a relevant role in the signaling leading to PTC death upon exposure to hypoxia [8], apoptotic bodies [9] or treatment with cisplatin [10], albumin [11] or leptin and gentamycin [12]. Therefore, it is possible that pro-inflammatory cytokines, through the COX-2 dependent stimulation of $PGE_2$ production, may contribute to PTC injury and, thus, to AKI development.

It is widely accepted that once $PGE_2$ is formed inside the cell, it is quickly released to the extracellular medium [13] to act in either an autocrine or paracrine manner through binding to specific G-protein coupled cell surface receptors (E series prostaglandin receptors EP1–4) [13]. Secreted $PGE_2$ must be taken up by cells for metabolism in order to prevent persistent and pathological inflammation. This task is mainly performed by the prostaglandin uptake transporter (PGT), which accounts for almost 100 % of $PGE_2$ internalization [14]. However, $PGE_2$ metabolism is not the unavoidable destiny of internalized $PGE_2$ because recent studies, have disclosed that intracellular $PGE_2$ ($iPGE_2$) has biological actions and that EP receptors which are located inside the cells might mediate the effects of $iPGE_2$ [15]. Therefore, besides its role in the cellular uptake of $PGE_2$ leading to its metabolic inactivation, PGT is now also considered to play a role in the delivery of secreted $PGE_2$ to its intracellular sites of action [15]. This view explains our previous findings showing that cisplatin-, apoptotic bodies- and hypoxia-induced PTC deaths is prevented by inhibition of PGT with bromocresol green or bromosulfophthalein as well as by antagonism of EP receptors [8–10].

Here we exposed human kidney-2 (HK-2) cells -an immortalized human PTC-derived cell line- to an inflammatory cytokine cocktail (to mimic systemic inflammation rather than induce one specific inflammatory pathway) as an in vitro model to investigate the therapeutic value of PGT targeting in the prevention of the deleterious effects of pro-inflammatory cytokines in PTC injury leading to AKI.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Reagents and antibodies were obtained from the following sources: 5′-bromo-2′-deoxyuridine (BrdU), celecoxib, anti-β-actin antibody, DAPI, bromosulfophthalein, crystal violet, fluorescein-labelled 4-kDa dextran and 70-kDa dextran, human collagen IV, anti-mouse IgG and anti-rabbit IgG peroxidase conjugated from Sigma Aldrich (Missouri, USA); antibodies anti-$PGE_2$, and anti-COX-2 from Abcam (Cambridge, UK); antibody anti-BrdU from BD Biosciences (California, USA); anti-mouse Alexa Fluor 594 IgG (H-L) and anti-mouse Alexa Fluor 488 IgG (H-L) from Jackson ImmunoResearch (Pennsylvania, USA); ProLong Gold Antifade Reagent® was from Invitrogen (Massachusetts, USA); bovine serum albumin (ChemCruz®), Phalloidin CruzFluor™ 594 conjugated and L798,106 from Santa Cruz (California, USA), TNF-α, IFN-γ, IL-1α, IL-1β and IL-2 were from Peprotech (London, UK). BCA Protein Assay Kit and Trizol reagent were from Thermo Fisher (Massachusetts, USA); reverse transcriptase and Supreme qPCR Green Master Mix were from NZYTECH (Lisboa, Portugal): AH6809, GW627368X, SC-19220 and PF-04419948 from Cayman Chemical (Michigan, USA); and Passive Lysis Buffer from Promega (Biotech Ibérica SL, Madrid, Spain).

### 2.2. Cell culture and experimental conditions

Human proximal tubular HK-2 cells were obtained from American Type Culture Collection (Rockville, MD). HK-2 cells were maintained in DMEM-F12 supplemented with 10 % fetal bovine serum, 1 % penicillin (10.000 units/ml)/streptomycin (10 mg/ml)/amphotherycin B (25 μg/ml) (Gibco, Thermo Fisher, Massachusetts, USA) and 1 % Insulin (10 mg/l)-Transferrine (5.5 mg/l) -Selenium (5 μg/l) (Sigma, Missouri, USA). The culture was performed in a humidified 5 % $CO_2$ environment at 37 °C. In all the experiments, cells were plated at 70 % confluence.

Cells were treated with a cytokine cocktail (TNFα: 50 ng/ml, IFNγ: 20 ng/ml, IL-1α: 10 ng/ml, IL-1β: 10 ng/ml, IL-2: 40 ng/ml). When necessary, HK-2 cells were pretreated with celecoxib (2 μM), bromosulfophthalein (25 μM), AH6809 (10 μM), GW 627368X (10 μM), SC-19220 (10 μM), PF-04419948 (1,9 μM) or L798,106 (1 μM) 1 h before the addition of the cytokine cocktail.

### 2.3. Crystal violet and trypan blue assays

Crystal violet is a dye that stains cell proteins and DNA in cells and as such can be used as a measure of adherent cell number. For this assay, the cells were seeded in 24-well plates ($1.5 \times 10^4$ cells/well) and 24 h later they were treated as described in Results. Afterwards, they were incubated for 10 min with crystal violet solution (0.25 % crystal violet and 20 % aqueous methanol) and the unbound dye was removed by washing the wells with water. Then, cells were photographed at 10× magnification using a Olympus IX-70 S22 inverted microscope. Finally, the bound dye was extracted from the adherent cells using 300 μl DMSO, and the absorbance of the solubilized dye was measured spectrophotometrically using a Synergy® HT ELISA plate reader (BioTek Vermont, US) at a wavelength of 595 nm. The absorbance results were interpolated to a calibration curve (optical density vs cell number) to obtain the number of cells in each assay.

Because viable (live) cells possess intact cell membranes, they are able to exclude "vital dyes" such as Trypan blue. This property allows for the discrimination between viable cells, which have a clear cytoplasm exclude and cells with damaged membrane, which have a blue cytoplasm. Once cells were treated as indicated in Results, the cells detached during experimental procedure were pelleted by centrifugation of the culture medium (500 ×*g*/5 min). Adherent cells were trypsinized and pelleted by centrifugation 5 min at 500 ×*g*. Pelleted cells were mixed after being resuspended in 0,4 % Trypan Blue stain and culture medium (1:1 *v*/v). Then, Trypan blue positive cells versus total cells were counted manually using a light microscope and a hemocytometer and the percentage of death cells was calculated.

### 2.4. Cell proliferation assay with BrdU (5′-Br-2′-deoxyuridine)

Detection newly synthesized DNA of actively proliferating cells was assessed by measuring BrdU incorporation into cells. For this purpose, cells were seeded in coverslips ($1.5 \times 10^4$ cells) and 24 h later they were treated as indicated in Results. Cells were then incubated for 2 h with 10 μM BrdU and they were fixed 70 % ethanol for 20 min. DNA was partially denatured at room temperature by incubation with 0.07 N NaOH (2 min), which was neutralized with 3 washes with PBS. Afterwards, cells were first incubated for 30 min at 37 °C with anti-BrdU (1:100 in 0,5 % Tween-PBS) and then with fluorescent secondary antibody anti-mouse Alexa Fluor 594 IgG (H-L) (1:800 in 0,5 % Tween-PBS). After 3 washes with PBS, cell nuclei were stained with DAPI (1 μg/ml in PBS) for 5 min and washed again three times with PBS. Finally, coverslips were mounted with ProLong Gold Antifade Reagent. DNA synthesis was estimated as the percentage of BrdU-positive cells, which was determined through manual count. To this end, cells in five fields were examined with a fluorescence microscope Olympus BX63 in a blind manner.

### 2.5. Immunofluorescence analysis

Cells were split on 12 $mm^2$ coverslips ($1.5 \times 10^4$ cells/coverslip) and incubated for 24 h before being treated as indicated in the Results

section. Afterwards, cells were fixed with 4 % paraformaldehyde during 20 min, permeabilized with 0.1 % Triton X-100 for 10 min and blocked with 4 % bovine serum albumin at room temperature. After three washes, coverslips were first incubated at 4 °C for 16 h with phalloidin (1:1000 dilution) or anti-$PGE_2$ (1:100 dilution) and then at room temperature for 1 h with anti-rabbit secondary antibodies Alexa-Fluor 488 IgG (H-L) (1:800 dilution). Cell nuclei were then stained with DAPI (1 μg/ml in PBS) for 5 min and washed three times with PBS. Finally, coverslips were mounted with ProLong and immunofluorescence detection was performed using a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), through the Confocal Microscopy Service (ICTS 'NANBIOSIS' U17) of the Biomedical Research Networking Centre on Bioengineering, Biomaterials, and Nanomedicine (CIBER-BBN at the University of Alcala, Madrid, Spain) (http://www.uah.es/enlaces/investigacion.shtm). $iPGE_2$-dependent immunofluorescence intensity was quantified after digital capture using Fiji software.

### 2.6. Protein isolation and Western blot analysis

Cells were plated in a six-well plated ($1.5 \times 10^5$ cells/well), incubated for 24 h and treated as indicated in Results. Then Passive Lysis Buffer with protease inhibitors was added and cells were harvested by scraping. Cells were kept on ice for 30 min to allow cell lysis and, after centrifugation at 5000 ×*g* for 10 min, the protein content in the cell lysates was quantified by BCA Protein Assay Kit. Proteins from cell lysates were denatured by addition of denaturing loading buffer (50 mM Tris (pH 6.8), 50 % glycerol, 0.125 % bromophenol blue, 15 % SDS and 25 % 2.5 M β-mercaptoethanol) and heating. Then, approximately 35 μg of protein were resolved by 8–15 % SDS–PAGE and blotted onto a 0.4 % or 0.2 % nitrocellulose membrane (Bio-Rad Laboratories, CA) during 2 h at 300 mA, at 4 °C, (25 mM Tris, 200 mM Glycine y 20 % methanol). Membranes were blocked for 1 h with 5 % powdered skimmed milk in T-TBS (2 mM Tris, 0.15 mM NaCl y 0.05 % de Tween-20) and incubated later for 16 h at 4 °C with primary antibodies: anti-COX-2 (1:1000, rabbit) or anti-β-actin antibody (1:10,000, mouse) as loading control. Finally, membranes were incubated for 1 h at room temperature with the corresponding secondary antiserum (1:4000) and the signals were detected with enhanced chemiluminescence reagent using Amersham Imager 680 QC. Quantification of band densities was performed using Fiji software.

### 2.7. RNA extraction and quantitative real-time PCR (q-RT-PCR)

Cells were plated in a six-well plated ($1.5 \times 10^5$ cells/well), incubated for 24 h and treated as indicated in Results. Total RNA was extracted using TRIZOL reagent. According to manufacturer's protocol. For cDNA synthesis, reverse transcription was performed using 1 μg of RNA and Reverse transcriptase via manufacturer's instruction. q-RT-PCR was performed using Supreme qPCR Green Master Mix (COX-2: 5′-CCAGAGCAGGCAGATGAAATA-3′ (Forward); 5′-CAGCATCGATGTCACCATAGAG-3′ (Reverse); and GADPH: 5′ CAAGGGCATCCTGGGCTAC 3′ (Forward); 3′-GCCCCAGCGTCAAAGGTGGA-5′ (Reverse)). The reaction was performed in 12 μl total volume. Relative COX-2 gene expression differences were calculated by the ΔΔCt method. GAPDH mRNA was used as endogenous control.

### 2.8. Cell detachment assay

6-well dishes were precoated with human collagen IV overnight at 4 °C to achieve a coating density of 13 μmol $cm^{-2}$. HK-2 cells were cultured in the collagen IV-plates and treated as indicated in Results. Then, the cells detached during the experimental procedure and the adherent cells (which were previously harvested by trypsinization) were separately pelleted by centrifugation of the culture medium (500 ×*g*/5 min), resuspended in 0.4 % Trypan Blue stain and culture medium (1:1 *v*/v) and counted manually using a light microscope and a hemocytometer. The number of detached cells for every experimental condition was calculated as the percentage of floating cells respect to the sum of the number of floating and attached cells and data were normalized with respect to the percent of detached cells found in the control.

### 2.9. Paracellular permeability

HK-2 cells were cultured on 24-well transwell dishes with a pore size of 0.4 μm (Corning Costar). Cells were allowed to form a tight monolayer for 3 days. Thereafter, cells were treated in the apical compartment (0.2 ml complete medium) as indicated in Results. Then cells were washed, and they were incubated for 24 h with fluorescein-labelled 4-kDa dextran or 70-kDa dextran (100 μg/ml; Sigma-Aldrich) in the apical compartment. Finally, samples were taken from the basolateral compartment, and both dextrans were analysed using a fluorescent plate reader (VICTOR X4, Perkin Elmer).

### 2.10. Statistical analysis

The results are expressed as the mean ± SD. They were subjected to one-way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons, or by two tail t-student analysis. The level of significance was set at $P < 0.05$. Each experiment was repeated at least three times.

## 3. Results

The present work is based on the hypothesis that inflammatory cytokines induce noxious, $iPGE_2$-dependent effects in human PTC. In order to check this hypothesis, we first asked whether exposure of HK-2 cells to a cytokine cocktail consisting of IL-1α, IL-1β, IL-2, TNF-α and IFN-γ (to mimic systemic inflammation rather than induce one specific inflammatory pathway) resulted in increased $iPGE_2$ levels in this human PTC line. The results shown in Fig. 1 a confirmed our hypothesis and further studies indicated that an increase in COX-2 expression (Fig. 1b) was likely responsible for the rise in the intracellular content of $PGE_2$ in HK-2 cells treated with the cytokine cocktail because the increase in $iPGE_2$ was prevented by COX-2 inhibitor celecoxib (Fig. 1c). Given that newly synthesized $PGE_2$ is immediately secreted to the extracellular medium, the increase in $iPGE_2$ in cytokine cocktail-treated cells requires in all probability the PGT-mediated reuptake of secreted $PGE_2$. In order to confirm this view, we investigated the effect of PGT inhibitor bromosulfophthalein on the rise in the intracellular content of $PGE_2$ in HK-2 cells treated with the combination of inflammatory cytokines. Our results (Fig. 1d) indicated that preincubation with the PGT inhibitor prevented the increase in $iPGE_2$ induced by cytokine treatment. In summary, the results shown in Fig. 1 imply that the inflammatory cytokine cocktail promotes a COX-2-, PGT-dependent increase in $iPGE_2$ in proximal tubular HK-2 cells.

Of note, extracellular $PGE_2$ also increased in a celecoxib-sensitive, bromosulfophthalein-insensitive manner in cells exposed to cytokines (Suppl. Fig. 1).

We next studied the effect of the inflammatory cytokines in the number of adherent HK-2 cells and found that there was a dramatic decrease in the cell number after 30 h incubation with the cytokine cocktail, as assessed by crystal violet assay (Fig. 2 upper panel). This effect was prevented by preincubation with PGT inhibitor bromosulfophthalein, which suggested that the decrease in cell number was most likely mediated by PGT-dependent uptake of $PGE_2$. In order to confirm that $PGE_2$ was actually involved, we investigated the effect of AH6809 and GW627368X, respective antagonists of EP1–3 and EP4 receptors, on the reduction of the cell number caused by the inflammatory cytokines. As shown in Fig. 2 lower panel, this effect was prevented by pre-incubation with AH6809 but not by GW627368X. Therefore, $PGE_2$ mediates the reduction in the cell number induced by

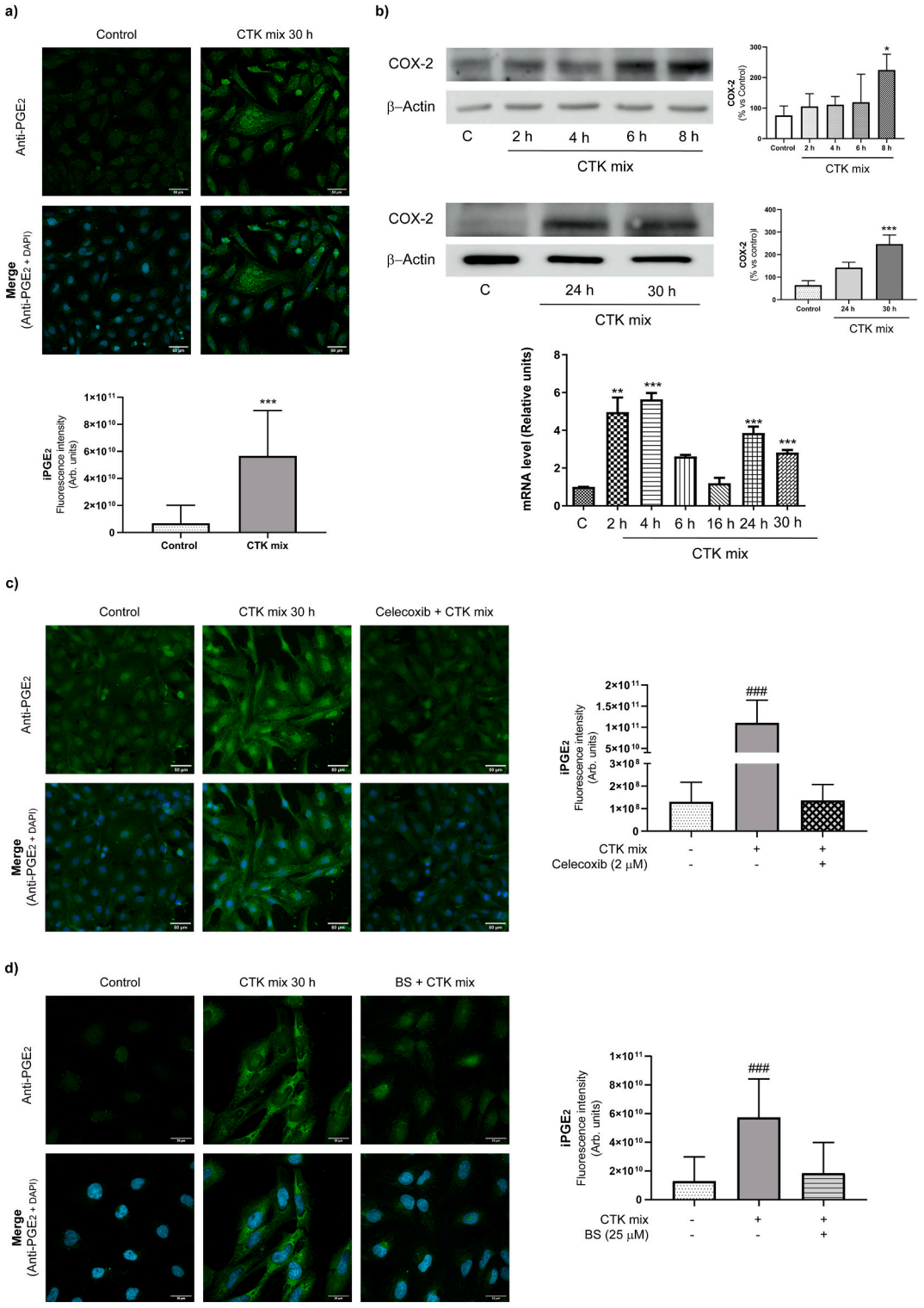


**Fig. 1.** Inflammatory cytokines increase COX-2 expression and $iPGE_2$ in a PGT-dependent manner in human renal proximal tubular HK-2 cells. a) *Increase in $iPGE_2$.* Upper panel: $iPGE_2$-dependent immunofluorescence (original magnification 25×) after incubation with inflammatory cytokines for 30 h. Lower panel: quantitative approach (Fiji software) to the images. b) *Increase in COX-2 expression.* COX-2 expression was assessed through Western blot analysis or q-RT-PCR analysis. Equal protein was confirmed by probing with an anti-β-actin antibody. Data in bars are % change over control of the densitometric analysis in which COX-2 expression was normalized to β-actin. Relative gene expression differences were calculated by the ΔΔCt method. GAPDH mRNA was used as an endogenous reference. c) COX-2 inhibitor celecoxib prevents cytokine-induced increase in iPGE2. d) *Cytokine cocktail-induced increase in $iPGE_2$ is prevented by PGT inhibitor bromosulfophthalein.* Left: $iPGE_2$-dependent immunofluorescence (original magnification 25×). Right: quantitative approach (Fiji software) to the images.
General information: 1) Cytokines cocktail (CTK mix) is composed of TNFα: 50 ng/ml; IFNγ: 20 ng/ml; IL-1α: 10 ng/ml; IL-1β: 10 ng/ml; IL-2: 40 ng/ml; bromosulfophthalein (BS) or Celecoxib were added to the incubation medium 1 h before the cytokines cocktail 2) Microphotographs are representative examples of three independent experiments. 3) Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments: *P < 0,05 vs control; **P < 0,01 vs control; ***P < 0,001 vs control; $^{###}$P < 0,001 vs other groups.

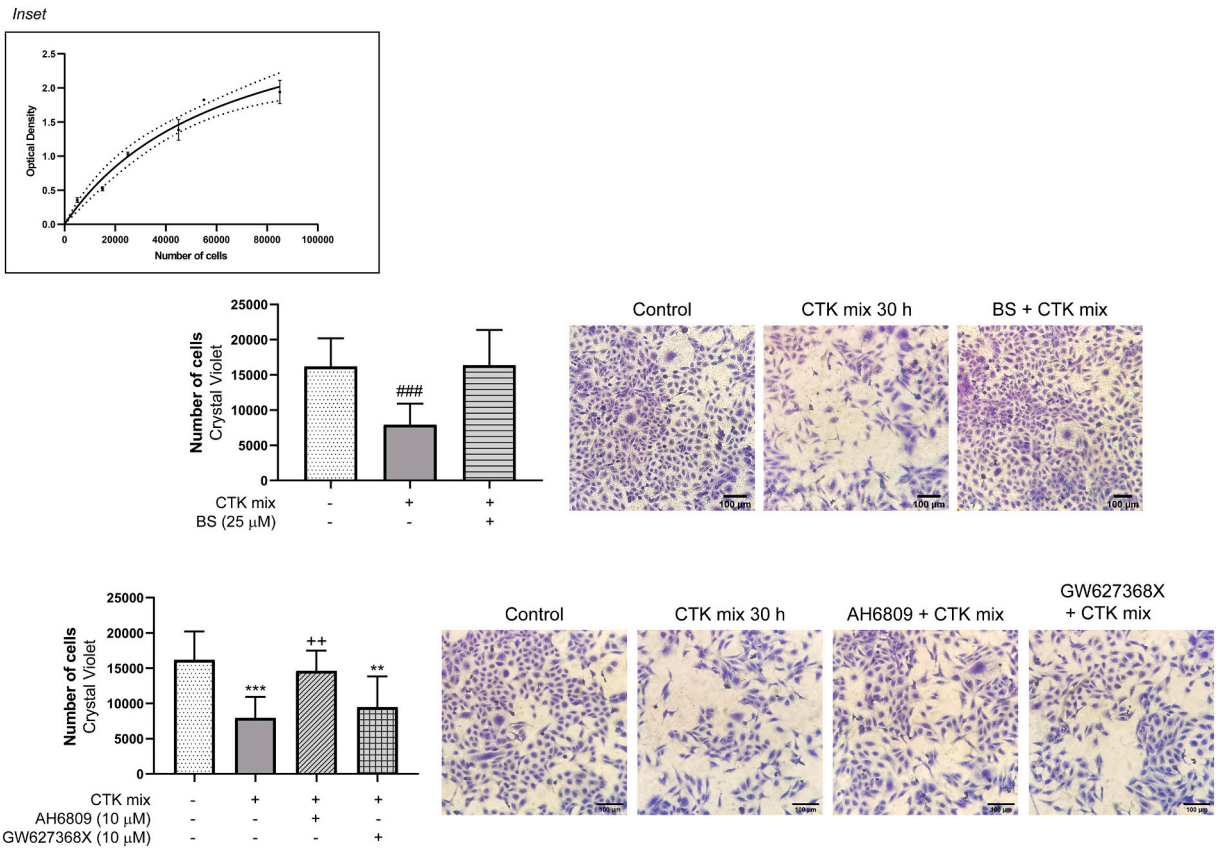


**Fig. 2.** Inflammatory cytokines induce a PGT-, EP receptor-dependent decrease in cell count (crystal violet assay) in human renal proximal tubular HK-2 cells. Cells were treated for 30 h with inflammatory cytokines (CTK mix: TNFα: 50 ng/ml; IFNγ: 20 ng/ml; IL-1α: 10 ng/ml; IL-1β: 10 ng/ml; IL-2: 40 ng/ml). PGT inhibitor bromosulfophthalein (BS) or EP receptor antagonists AH6809 and GW627368X, were added to the incubation medium 1 h before the cytokine cocktail. Left: The number of adherent cells was calculated by crystal violet assay as indicated in Materials and methods through a calibration curve (inset). Right: Microphotographs (original magnification 10×) are representative examples of three independent experiments Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments: $^{\#\#\#}P < 0{,}001$ vs other groups, ***P < 0,001 vs control; **P < 0,01 vs control; $^{++}P < 0{,}01$ vs CTK mix.

the mixture of cytokines in an EP1–3 receptor-, PGT-dependent manner.

Since cell proliferation and cell death may contribute to changes in cell number, we analysed the impact of inflammatory cytokines in both parameters. To this end, HK-2 cells were incubated with the cytokine cocktail and cell proliferation and cell viability were respectively assessed by BrdU incorporation and trypan blue assays. As shown in Fig. 3 cytokine cocktail-treated HK-2 cells exhibited an important reduction in both cell proliferation and cell viability. Again, pre-incubation with PGT inhibitor bromosulfophthalein or with EP-1-3 receptor antagonist AH6809 or with specific antagonists of EP1, EP2 or EP3 receptors (SC-19220, PF-04419948, L798, 106, respectively), but not with EP4 receptor antagonist GW627368X, resulted in prevention of the changes induced by inflammatory cytokines. These results suggest in the first place that both, decreased cell proliferation and enhanced cell death may contribute to the reduction in number of adherent HK-2 cells found in cytokine cocktail-treated cells (Fig. 2a). In the second place these results indicate that, similar to what happened with HK-2 cell number, $PGE_2$ also mediates the reduction in cell proliferation and cell viability induced by the mixture of inflammatory cytokines in an EP1–3 receptor-, PGT-dependent manner.

As previously reported, inflammatory cytokines such as TNF-α, IL-1α and IFN-γ induce proximal tubular epithelial cell detachment [4]. This is relevant because acute tubular injury may be associated with PTC detachment into the lumen leading to back-leakage of glomerular ultrafiltrate and tubule obstruction [16]. Having into account this background, we asked whether the inflammatory cytokine cocktail also induced HK-2 cell detachment from collagen IV (a major constituent of the kidney tubular basement membrane [17]) and, if so, whether this effect was dependent on $iPGE_2$.Our results confirmed that the combination of inflammatory cytokines determined a PGT-dependent increase in cell shedding as indicated by its prevention by bromosulfophthalein (Fig. 4 upper panel). Cytokine-induced HK-2 detachment from collagen IV was partially prevented by antagonists of EP receptors (Fig. 4 lower panel). These data suggest that inflammatory cytokines may increase the degree of PTC detachment from collagen IV and point out that PGT plays a critical role in this effect, which is mediated, at least in part, by $iPGE_2$.

The loss of adhesion induced by the combination of cytokines implies significant changes in epithelial cell morphology and monolayer integrity. In order to explore both aspects we used, respectively, phalloidin staining to monitor the changes in F-actin cytoskeleton and FITC-labelled dextrans to estimate the changes in paracellular permeability. Upon 30 h cytokine exposure, the morphological features of HK-2 cells changed dramatically -which is coincident with a previous study [4]- and they exhibited a significant reorganization of cortical actin cytoskeleton related to a significant loss of F-actin-based structures (Fig. 5a). As a consequence, the intercellular contacts appeared disrupted at several sites with the appearance of gaps between cells so that it resulted in loss of the monolayer integrity. These changes were prevented with the pretreatment with bromosulfophthalein and AH6809 but not with GW627368X. The estimation of the epithelial barrier permeability allowed us to assess the functional consequences of these morphological changes in HK-2 cells: as shown in Fig. 5b, the mixture of inflammatory cytokines induced a statistically significant increase in apical-to-basolateral transepithelial flux of FITC-labelled dextrans (70 and 4 kDa) as compared to control HK-2 cells. Preincubation with PGT inhibitor bromosulfophthalein or with antagonist of EP1–3 receptors

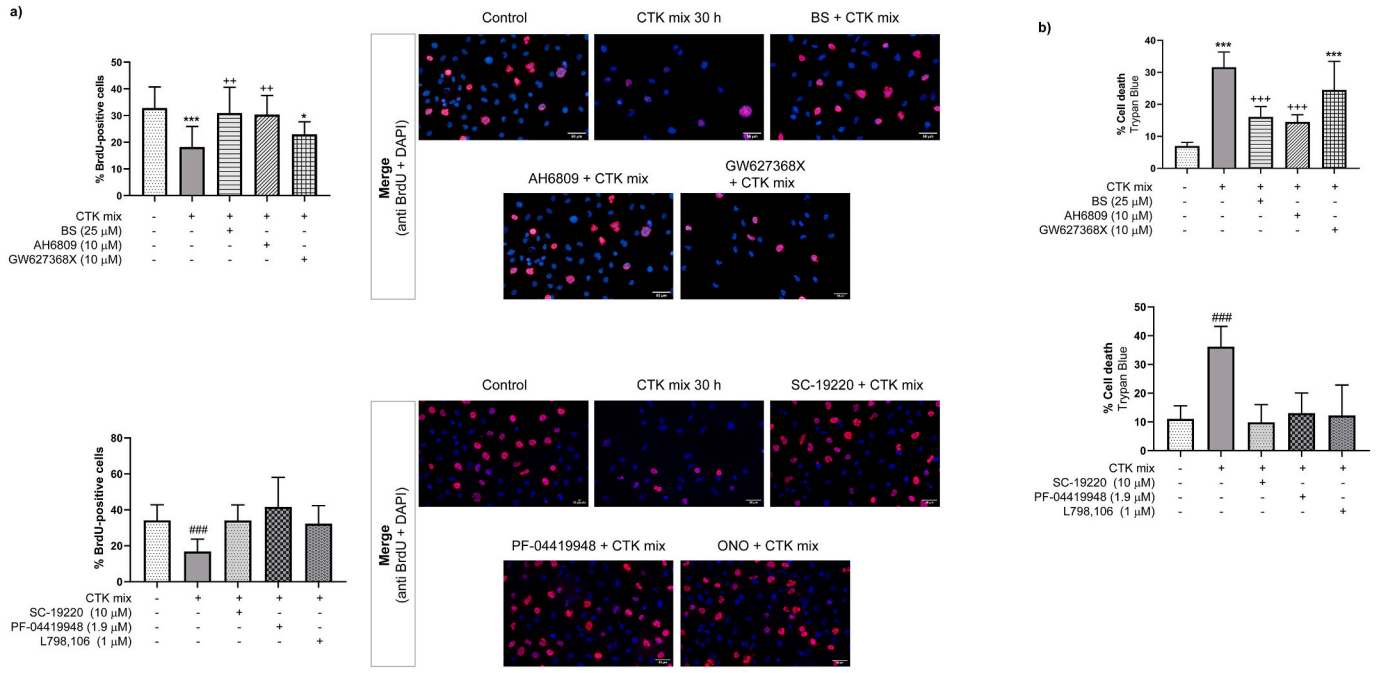


**Fig. 3.** Inflammatory cytokines induce a PGT-, EP receptor-dependent decrease in cell proliferation and cell viability in human renal proximal tubular HK-2 cells. Cells were treated for 30 h with inflammatory cytokines (CTK mix: TNFα: 50 ng/ml; IFNγ: 20 ng/ml; IL-1α: 10 ng/ml; IL-1β: 10 ng/ml; IL-2: 40 ng/ml). PGT inhibitor bromosulfophthalein (BS) or EP receptor antagonists AH6809, GW627368X, SC-19220, PF-04419948, L798,106, were added to the incubation medium 1 h before the cytokine cocktail a) *Cell proliferation* (BrdU incorporation assay). The percentage of BrdU-positive cells was determined through manual count in five fields Microphotographs (original magnification 20×) are representative examples of three independent experiments in which cell nuclei appear blue colour (DAPI stain) and BrdU positive cells appear red. b) *Cell viability* (trypan blue assay). Trypan blue positive cells versus total cells were counted manually using a light microscope and a hemocytometer and the percentage of death cells was calculated. Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments $^{\#\#\#}P < 0{,}001$ vs other groups; *P < 0,05 vs control; ***P < 0,001 vs control; $^{++}P < 0{,}01$ vs CTK mix; $^{+++}P < 0{,}001$ vs CTK mix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AH6809, but not with EP4 receptor GW627368X, resulted in full prevention of cytokine-induced increase in permeability to 70 kDa dextran and in partial prevention in the case of 4 kDa dextran. These results suggest that inflammatory cytokines, through PGT-, iPGE$_2$-dependent mechanisms may disrupt the paracellular diffusion barrier in PTC so that proximal tubules may become leakier.

## 4. Discussion

Acute kidney injury associates with a strong local and systemic inflammatory response and it is characterized by some degree of PTC injury. The increased release of inflammatory mediators by immune and resident cells has been shown to contribute to PTC damage leading to kidney failure [2]. Here we show that human proximal tubular HK-2 cells suffer detrimental consequences upon exposure to a mixture of pro-inflammatory cytokines (consisting of IL-1α, IL-1β, IL-2, TNF-α and IFN-γ) which have been connected with acute tubular cell injury and dysfunction [1]. In our experimental setting, the cytokine cocktail also induced an increase in iPGE$_2$ so that its harmful effects on HK-2 cells were prevented by antagonism of EP1–3 receptors and by the inhibitor of the prostaglandin transporter PGT bromosulfophthalein (which also avoided the increase in iPGE$_2$). These results underscore the role of iPGE$_2$ in the noxious effects of the inflammatory cytokines on PTC.

A fundamental requirement for the physiological performance of organs is the formation of diffusion barriers that separate and maintain compartments of different structure. Epithelial layers, such as the tubular epithelium in the kidney, form a barrier that mediates controlled transcellular and paracellular transport of ions and various substances [18]. PTC are attached to the basal membrane (where collagen IV is the major component) and they are joined to each other via a set of intercellular junctions, both features being important for proximal tubule barrier formation and function. Our results have demonstrated that the combination of inflammatory cytokines determines several PGT-, EP1–3 receptor-dependent changes in HK-2 cells such are loss of adherent cells, increase in cell shedding from collagen IV and cell death, enhanced apical-to-basolateral transepithelial flux of FITC-labelled dextrans and significant reorganization of cortical actin cytoskeleton resulting in loss of the monolayer integrity and the appearance of gaps between cells (Figs. 2-5). Interestingly, previous studies in cultured PTC have disclosed that mixtures of two or three inflammatory cytokines such as IL-1α, IL-1β, IL-2, TNF-α and IFN-γ (i.e., the same cytokines tested in our work) induce changes similar to those described by us [3–5]. Taken together, the results shown in Figs. 2 to 5 suggest that the inflammatory cytokines tested might disrupt the specific barrier and permeability characteristics of the proximal tubule epithelium through deranging PTC adhesion to the tubule basal membrane and PTC intercellular junctions, as well as through diminishing PTC viability. In addition, PTC detachment into the lumen may lead to back-leakage of glomerular ultrafiltrate and tubule obstruction [16]. Finally, inflammatory cytokines, through diminishing PTC proliferation, might also compromise the repair of proximal tubules given that PTC proliferation is required for restoration of the renal tubular epithelium following sublethal kidney injury, [19] Therefore, the combined effects on the proximal tubule of cytokines such as of IL-1α, IL-1β, IL-2, TNF-α and IFN-γ might contribute to the pathogenesis of AKI.

It is commonly assumed that PGE$_2$ is a paracrine/autocrine mediator and that as such it must be secreted to the extracellular medium to exert its actions via plasma membrane EP receptors. Nevertheless, our results indicate that the re-uptake of PGE$_2$ by PGT (through which PGE$_2$ becomes iPGE$_2$) plays a critical role in the effects of the cytokine cocktail on HK-2 cells so that they are prevented by PGT inhibitors (Figs. 2-5 and Suppl. Fig. 2). The fact that antagonism of EP1–3 receptors also

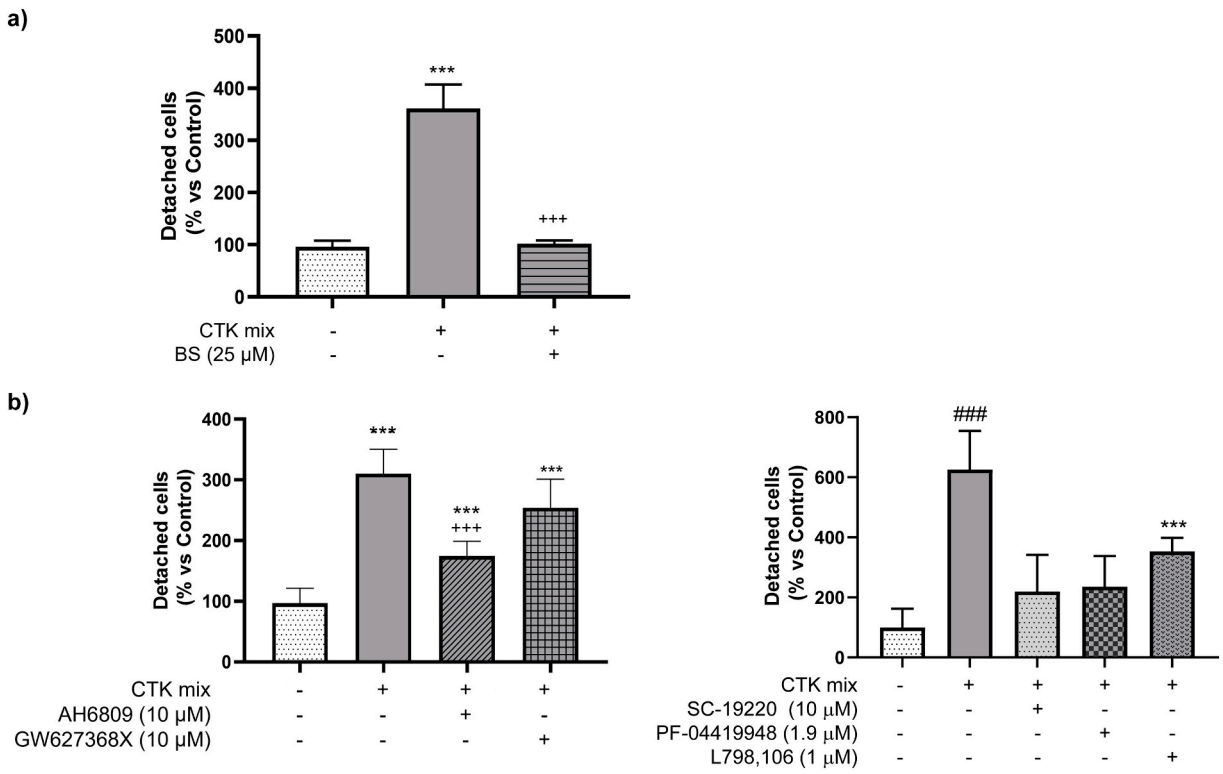


**Fig. 4.** Inflammatory cytokines induce a PGT-, EP receptor-dependent increase in cell detachment from collagen IV in human renal proximal tubular HK-2 cells. Cells were treated for 24 h with inflammatory cytokines (CTK mix: TNFα: 50 ng/ml; IFNγ: 20 ng/ml; IL-1α: 10 ng/ml; IL-1β: 10 ng/ml; IL-2: 40 ng/ml. a) PGT inhibitor bromosulfophthalein (BS) or b) EP receptor antagonists AH6809, SC-19220, PF-04419948, L798,106 and GW627368X, were added to the incubation medium 1 h before the cytokine cocktail. The percentage of detached cells was calculated as indicated in Material and Methods. Bars and error bars in graphs: Each bar represent the mean ± SD of 3 different experiments: $^{###}P < 0{,}001$ vs other groups; $^{***}P < 0{,}001$ vs control; $^{+++}P < 0{,}001$ vs CTK mix.

prevented these effects, suggests that they are due to the activation by $iPGE_2$ of an intracellular subset of EP receptors so that PGT would be required to deliver secreted $PGE_2$ to this intracellular site of action. In support of this hypothesis, we have observed in HK-2 cells both plasma membrane and intracellular EP receptors (Suppl. Fig. 3). However, further studies are required to elucidate the precise role of intracellular EP receptors in the current results because we do not have data which prove that EP receptor antagonists were taken up by cells and reached a significant concentration inside to block the intracellular EP1–3 receptors. Furthermore, even if those data were available, we could not rule out a role of plasma membrane receptors in the EP receptor antagonist-sensitive effects of inflammatory cytokines on HK-2 cells.

COX inhibitors are criticized for causing renal unwanted side effects, which is a major obstacle for use in the prevention and treatment of inflammation associated kidney injury. In this context, targeting downstream signaling pathways of $PGE_2$ may represent an attractive new therapeutic strategy. Taking into account the relevant role of $iPGE_2$ in the noxious effects of inflammatory cytokines on proximal tubular HK-2 cells, we propose to test inhibitors of PGT as a novel therapeutic approach to prevent AKI induced by sepsis, renal ischemia/reperfusion and other pathological conditions in which production of inflammatory cytokines may contribute to proximal tubular damage. In this regard, bromosulfophthalein is probably the best candidate to start with in terms of drug safety because it has been widely used in the past in liver function tests [20].

## 5. Conclusion

The present study collectively demonstrates the role of PGT and $iPGE_2$ in inflammatory cytokine-induced HK-2 cell damage and outlines different mechanistic pathways implicated such as decreased cell proliferation, increased paracellular permeability and cell death, and loss of both adherent cells to collagen IV and monolayer integrity. In addition, the current results of this study highlight the prevention afforded against these inflammatory cytokine-induced changes by PGT inhibitor bromosulfophthalein, which has long been widely used in the past in liver function tests. Based on our findings, bromosulfophthalein could be used -after further validation in animal models and clinical studies- as an effective and safe agent to prevent inflammatory cytokine-induced PTC injury leading to AKI.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.121260.

## CRediT authorship contribution statement

**Julia Yago-Ibáñez:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Laura Muñoz-Moreno:** Methodology, Software, Formal analysis, Writing – original draft, Writing – review & editing. **Beatriz Gallego-Tamayo:** Methodology, Software, Formal analysis, Writing – original draft, Writing – review & editing. **Francisco Javier Lucio-Cazaña:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration. **Ana Belén Fernández-Martínez:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

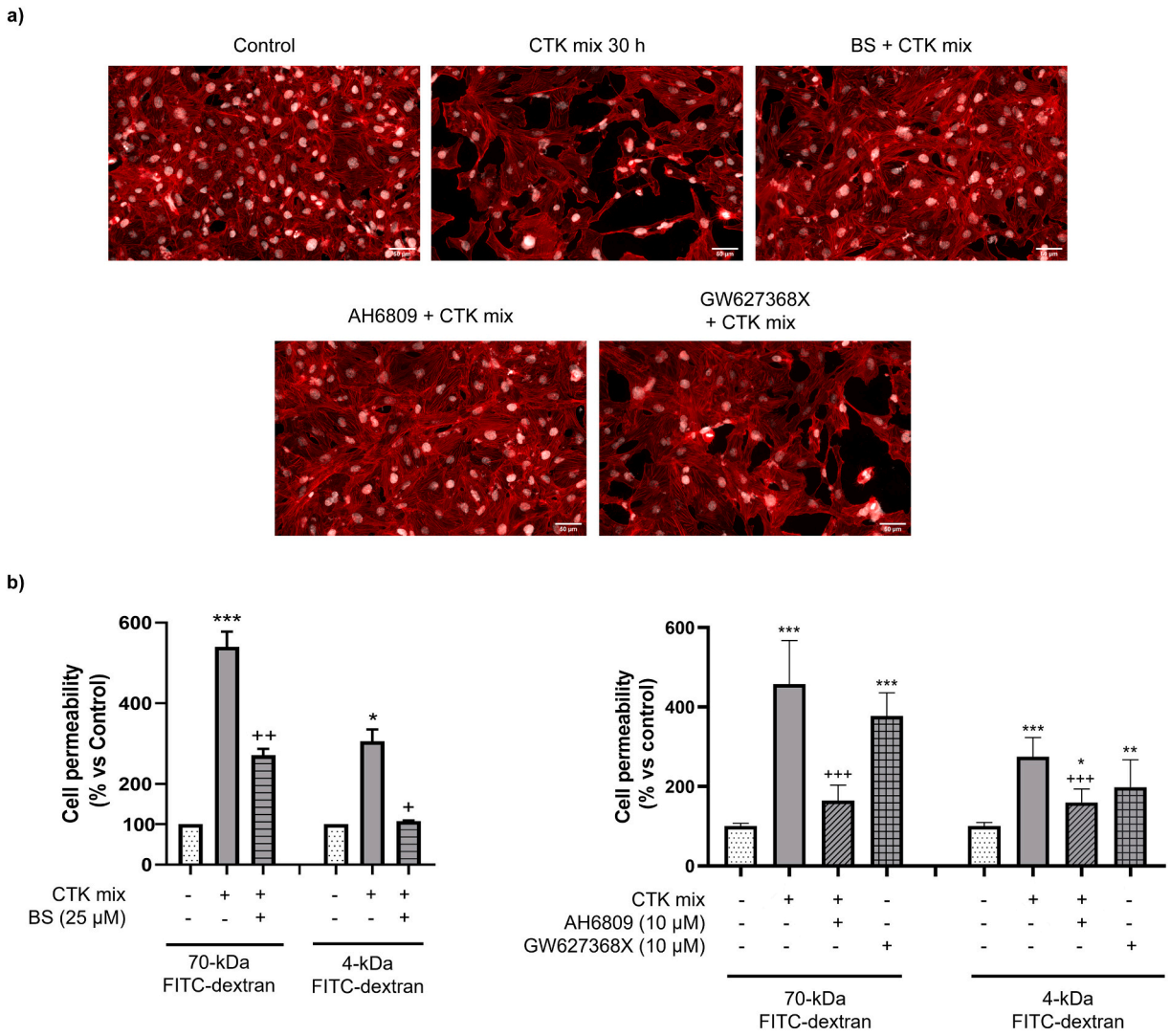


**Fig. 5.** Inflammatory cytokines induce changes in F-actin cytoskeleton and an increase paracellular permeability in human renal proximal tubular HK-2 cells in a PGT-, EP receptor-dependent manner. Cells were treated for 24 h with inflammatory cytokines (CTK mix: TNFα: 50 ng/ml; IFNγ: 20 ng/ml; IL-1α: 10 ng/ml; IL-1β: 10 ng/ml; IL-2: 40 ng/ml. PGT inhibitor bromosulfophthalein (BS) or EP receptor antagonists AH6809 and GW627368X, were added to the incubation medium 1 h before the cytokine cocktail. a) Changes in F-actin cytoskeleton organization, cell size and monolayer integrity. Representative microscopy images of phalloidin staining showing actin cytoskeleton reorganization and loss of the monolayer integrity (as evidenced by the gaps between cells) in HK-2 cells exposed to the cytokine mixture. b) Paracellular flux of 4 kDa- or 70 kDa-FITC dextrans across HK-2 cell monolayers grown in Transwell membranes was performed as described under Materials and Methods. The amount of FITC-dextran diffused to the basolateral side of the cytokine-treated monolayer was normalized by the average value obtained from control HK-2 cells. Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments: $^{*}P < 0{,}05$ vs control; $^{**}P < 0{,}01$ vs control; $^{***}P < 0{,}001$ vs control; $^{+}P < 0{,}05$ vs CTK mix; $^{++}P < 0{,}01$ vs CTK mix; $^{+++}P < 0{,}001$ vs CTK mix.

## Data availability statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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