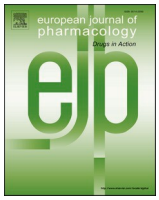
European Journal of Pharmacology 962 (2024) 176199

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar



Cyclooxygenase-2 inhibition prevents renal toxicity but not hypertension during sunitinib treatment

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ARTICLE INFO

Keywords:
Cardio-oncology
Angiogenesis inhibitors
Hypertension
Renal toxicity
Cyclooxygenase-2
Prostanoids

ABSTRACT

Background: Anticancer angiogenesis inhibitors cause hypertension and renal injury. Previously we observed in rats that high-dose aspirin (capable of blocking cyclooxygenase (COX)-1 and-2) was superior to low-dose aspirin (blocking COX-1 only) to prevent these side-effects during treatment with the angiogenesis inhibitor sunitinib, suggesting a role for COX-2. High-dose aspirin additionally prevented the rise in COX-derived prostacyclin (PGI_2). Therefore, we studied the preventive effects of selective COX-2 inhibition and the hypothesized contributing role of PGI_2 during angiogenesis inhibition.

Methods: Male WKY rats received vehicle, sunitinib ((SU), 14 mg/kg/day) alone or combined with COX-2 inhibition (celecoxib, 10 mg/kg/day) or a PGI_2 analogue (iloprost, 100 μg/kg/day) for 8 days (n = 8–9 per group). Mean arterial pressure (MAP) was measured via radiotelemetry, biochemical measurements were performed via ELISA and vascular function was assessed via wire myography.

Results: SU increased MAP (17±1mmHg versus 3±1mmHg after vehicle on day 4, $P < 0.002$), which could not be significantly blunted by celecoxib (+12±3mmHg on day 4, $P = 0.247$), but was temporarily attenuated by iloprost (treatment days 1 + 2 only). Urinary PGI_2 (996 ± 112 versus 51 ± 11ng/24h after vehicle, $P < 0.001$), but not circulating PGI_2 increased during SU, which remained unaffected by celecoxib and iloprost. Celecoxib reduced sunitinib-induced albuminuria (0.36 ± 0.05 versus 0.58 ± 0.05mg/24h after SU, $P = 0.005$). Wire myography demonstrated increased vasoconstriction to endothelin-1 after SU (Emax $P = 0.005$ versus vehicle), which remained unaffected by celecoxib or iloprost.

Conclusion: Selective COX-2 inhibition ameliorates albuminuria during angiogenesis inhibition with sunitinib, which most likely acts independently of PGI_2. To combat angiogenesis inhibitor-induced hypertension, dual rather than selective COX-1/2 blockade seems preferential.

1. Introduction

Angiogenesis inhibitors are effective anti-cancer agents used for the treatment of a wide variety of solid malignancies, but their clinical use can be hampered by serious treatment-induced hypertension and renal injury (van Dorst et al., 2021). Various factors have been demonstrated to contribute to these toxicities, including increased endothelin-1 (ET-1) signaling and vasoactive cyclooxygenase (COX)-derived prostanoids (Kappers et al., 2011; Mirabito Colafella et al., 2020, 2022). Although antagonists of the ET_A receptor were highly effective in rats to prevent toxicity induced by the multitargeted angiogenesis inhibitor sunitinib (Mirabito Colafella et al., 2020), their clinical translation is limited by side-effects and costs. Therefore, alternative preventive and clinically applicable strategies are urgently needed.

Previously, our group demonstrated that high-dose aspirin (resulting in dual inhibition of the COX-1 and COX-2 isoforms) was superior to low-dose aspirin (resulting in selective COX-1 inhibition) with regard to

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https://doi.org/10.1016/j.ejphar.2023.176199
Received 17 August 2023; Received in revised form 9 November 2023; Accepted 9 November 2023
Available online 27 November 2023

Non-standard abbreviations and acronyms

AUC	area under the curve
COX	cyclooxygenase
ELISA	enzyme-linked immunosorbent assay
EP3	prostaglandin E type 3 receptor
ET-1	endothelin-1
FITC	fluorescein-isothiocyanate
GFR	glomerular filtration rate
HR	heart rate
MAP	mean arterial pressure
PGE_2	prostaglandin E2
PGI_2	prostacyclin
TP	thromboxane receptor
TXA_2	thromboxane

the prevention of sunitinib-induced hypertension and, in particular, albuminuria (Mirabito Colafella et al., 2022). Although these observations point towards a major contribution of COX-2 to sunitinib-induced toxicity, its exact role independently of COX-1 remains to be determined. Interestingly, COX-2 has been shown to enhance vascular contraction upon ET_A receptor stimulation (Zhou et al., 2006) and ET-1 can in turn induce COX-2 expression (Lin et al., 2013). This ET-1 – COX-2 crosstalk additionally puts COX-2 inhibition forward as a novel strategy for the prevention of angiogenesis inhibitor-induced toxicities. We hypothesize that selective COX-2 inhibition would be an effective strategy to mitigate sunitinib-induced hypertension and renal injury.

COX-2 is involved in the formation of vasoactive prostanoids, mainly prostacyclin (PGI_2) and prostaglandin E2 (PGE_2) (Brock et al., 1999). Previously it was observed that sunitinib increased the circulating and urinary levels of PGI_2, but not PGE_2, and that urinary PGI_2 excretion was normalized during co-treatment with high-dose aspirin (Mirabito Colafella et al., 2022). Since PGI_2 is generally regarded as a potent vasodilator, an upregulation of PGI_2 may (initially) acts as a compensatory mechanism in the context of angiogenesis inhibition. In addition, PGI_2 is delicately balanced with the vasoconstrictor thromboxane (TXA_2) to maintain cardiovascular homeostasis and vascular tone (Atallah et al., 2017; Mirabito Colafella et al., 2019). Interestingly, previous studies have indicated that PGI_2 in excessive concentrations can also paradoxically induce vasoconstriction by acting as an endothelium-derived contracting factor (Vanhoutte, 2011; Vanhoutte and Tang, 2008). This is proposed to be predominantly mediated via stimulation of EP3 and TP receptors on vascular smooth muscle cells (Li et al., 2017; Liu et al., 2012). We therefore hypothesize that excessive COX-2-derived PGI_2 generation contributes to angiogenesis inhibition-induced toxicity.

In this study, we aimed to investigate the role of both COX-2 and PGI_2 in sunitinib-induced hypertension and renal injury by co-treatment of sunitinib with a selective COX-2 inhibitor (celecoxib) or a PGI_2 analogue (iloprost) in male Wistar Kyoto (WKY) rats. This is a well-established model of angiogenesis inhibitor-induced hypertension and kidney damage, which mimics the toxicity observed in the clinical situation (Lankhorst et al., 2015; Mirabito Colafella et al., 2020, 2022). We hypothesized that celecoxib would prevent, whereas iloprost would worsen sunitinib-induced hypertension and renal injury.

2. Materials and methods

2.1. In vivo study

All animal experiments were performed under the regulation and approval of the Animal Welfare Committee of the Erasmus MC (protocol number 16-511-04). Male, 10-week-old WKY rats (240–280 g) were purchased from Janvier Labs, Le Genest-Saint-Isle, France and housed in individual cages in an experimental room with temperature maintained at 21–22 °C on a 12-h light/dark cycle. Rats had *ad libitum* access to standard laboratory chow and water throughout the entire study period. After a 10-day acclimatization period, rats were anesthetized using isoflurane (2–4%) and a radiotelemetry probe (HD-S10, Data Sciences International, MN, USA) was implanted into the abdominal aorta for arterial blood pressure recordings for 10 s every 10 min. Analgesia (buprenorphine 0.05 mg/kg s.c.; RB Pharmaceuticals, Chennai, India) was administered prior to surgery and for 2 days afterwards. Following a 10-day recovery period, glomerular filtration rate (GFR) was measured via the transcutaneous decay of FITC-sinistrin, and rats were housed in metabolic cages for 48 h (first 24 h for acclimatization and second 24 h for urine collection) with free access to food and water. Subsequently, rats were acclimatized to oral gavage by administration of 0.5 mL drinking water for 7 days. Baseline blood pressure and heart rate values were obtained by averaging the measurements on the final 3 days of this training period. Subsequently, rats were randomized into different treatment groups (n = 8–9 per group): 1. vehicle (oral gavage), 2. sunitinib (14 mg/kg/day, oral gavage), 3. sunitinib + celecoxib (selective COX-2 inhibitor, 10 mg/kg/day, oral gavage), and 4. sunitinib + iloprost (PGI_2 analogue, 100 μg/kg/day, subcutaneous infusion). Iloprost was administered via continuous subcutaneous infusion by osmotic minipumps (Alzet®, model 2ML4, Durect Corp, Cupertino, CA, USA) implanted on the first day of treatment under isoflurane (2–4%) anesthesia. Animals from other groups underwent sham implantation by creation of a subcutaneous pocket and all animals received a single dose of analgesia afterwards. GFR was measured on day 7 and rats were housed in metabolic cages for 24-h urine sample collection afterwards. On day 8, rats were anesthetized by isoflurane (2–4%) and sacrificed by exsanguination via portal vein puncture. Blood was supplemented with EDTA and centrifuged at 13000 RPM for 10min to obtain plasma. Kidneys, heart, liver, spleen and abdominal aorta were rapidly excised and fixated in paraformaldehyde for histological analysis or snap frozen in liquid nitrogen for determination of protein expression. Iliac arteries were isolated for vascular function studies.

2.2. Preparation and dosage of compounds

The content of sunitinib L-malate capsules (Sutent®, Pfizer, equivalent to 50 mg sunitinib), obtained from patients with cancer who discontinued treatment, was dissolved in a final concentration of 14 mg/kg per 0.5 mL solution (daily treatment volume) in sterile water, HCl 0.1N, polyethylene glycol 300 (10%), polysorbate 80 (0.5%), and 0.1N NaOH to adjust pH to 3.3–3.5, identical to a previous study (Mirabito Colafella et al., 2020). The administered dosage of 14 mg/kg/day was based on previous experiments in the same animal model during which sunitinib plasma measurements were performed (Lankhorst et al., 2014, 2015; Mirabito Colafella et al., 2020). Celecoxib was purchased from Sigma-Aldrich and dissolved in DMSO (2%), polyethylene glycol 300 (30%), polysorbate 80 (5%) and dH_2O (63%). Iloprost was purchased from MedChemExpress and dissolved in DMSO (10%), polyethylene glycol 300 (40%), polysorbate 80 (5%), and NaCl (45%), according to the manufacturer's instructions. The administered dosage of celecoxib was chosen to establish a balance between sufficient COX-2 blockade and minimization of its possible prohypertensive effects (Muscará et al., 2000), but also to limit possible effects of (high doses of) celecoxib on renal and liver function (Koçkaya et al., 2010). The dosage of iloprost was based on a previous study which demonstrated the beneficial effects of iloprost (Zlatnik et al., 1999) on blood pressure levels in an animal model for preeclampsia. All other compounds were purchased from Sigma-Aldrich unless stated otherwise. Administration of 0.5 mL of drinking water via oral gavage was used as vehicle.

2.3. Ex vivo vascular function

At the end of the treatment period, wire myography was used to

assess the possible consequences of the *in vivo* treatment on vascular function. For this, iliac arteries were isolated and vascular function was assessed in response to the vasoconstrictor ET-1 in the absence or presence of ET_A (BQ123; 10^{-6}M) or ET_B (BQ788, 10^{-6}M) receptor blockade (30 min pre-incubation) in isolated iliac segments as described previously (Mirabito Colafella et al., 2020). In additional iliac segments, vasoconstriction responses were assessed in response to iloprost in the absence or presence of blockade of the prostaglandin E type 3 receptor (EP3) or thromboxane receptor (TP) via 30 min pre-incubation of L-798106 (10^{-6}M) or terutroban (10^{-6}M), respectively. All data were recorded and analyzed using the LabChart data acquisition system (AD Instruments Ltd, Oxford, UK).

2.4. Biochemical measurements

Plasma creatinine concentration was measured at the Clinical Chemical Laboratory of the Erasmus University Medical Center, Rotterdam. Plasma and urinary levels of PGI_2 (via 6-keto-PGF_{1a} ELISA Kit ADI-900-004, Enzo Life Sciences, detection limit: 1.40 pg/ml), TXA_2 (via TXB_2 ELISA Kit ADI-900-002, Enzo Life Sciences, detection limit: 10.54 pg/ml), ET-1 (Quantikine ELISA Kit DET100, R&D Systems, detection limit: 0.207 pg/ml), and albuminuria (ELISA Kit ab108789), Abcam, Cambridge, UK) were determined using a chemiluminescent enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.5. Glomerular filtration rate

Rats were anesthetized using isoflurane (2–4%) and a small patch of the fur of the back was removed using an electric shaver and application of depilatory cream for 6 min. Subsequently, a non-invasive clearance kidney fluorescent detection device was attached to the shaved area and fluorescein-isothiocyanate (FITC)-labelled sinistrin was administered via the tail vein (0.24 mg/kg, bolus injection). After 2 h, rats were briefly anesthetized again to remove the detection device and a partner software (Mannheim Pharma & Diagnostics GmbH, Mannheim, Germany) was used to calculate the elimination half-life ($t_{1/2}$) of sinistrin by generation of the elimination kinetics curve. The GFR was calculated by the validated formula for rats (Schock-Kusch et al., 2009):

GFR (mL/min per 100 g body weight (BW)) = 31.26 (mL / 100 g BW) / $t_{1/2}$ FITC-sinistrin (minutes)

2.6. Aortic and renal protein expression

Aortic and renal protein abundance of COX-1 and COX-2 were determined using Western blotting. Whole kidney and aorta samples were homogenized, lysed and centrifuged at 6000 RPM for 15 min at 4 °C. Subsequently, protein (40 μg) was separated by electrophoresis on a polyacrylamide gel and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with 5% bovine serum albumin in TBS solution containing 0.1% Tween-20. Membranes were then incubated with specific antibodies overnight at 4 °C. Membranes were washed three times with TBS-Tween20 and incubated with infrared dye-labelled secondary antibodies for 1h at room temperature. Signals were detected by chemiluminescence (Clarity Western ECL substrate; Bio-Rad, Hercules, CA, USA) and quantified using Image Studio Lite software. Results were normalized to GAPDH expression, are expressed in arbitrary units and were statistically compared with sunitinib only group. Expression of GAPDH was determined on the samples loaded on the COX-1 gels. Membranes were incubated with an antibody for either COX-1 (anti-COX-1 (1:1000, #9896, Cell Signaling Technology, Danvers, Massachusetts, USA)) or COX-2 (anti-COX-2 (1:1000, #12282, Cell Signaling Technology, Danvers, Massachusetts, USA)).

2.7. Statistical analyses

Data are presented as mean ± SEM unless indicated otherwise. Non-normally distributed data were log-transformed before statistical analysis. Statistical analyses between groups was performed by one-way ANOVA followed by Holm-Sidak's post hoc test. Blood pressure and heart rate were compared between groups by repeated measures ANOVA followed by Dunnett's multiple comparisons. For correlation analyses, the Pearson *r* correlation coefficient and the Spearman's rank correlation coefficient were used in case of normally and non-normally distributed data, respectively. GraphPad Prism Version 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Iloprost, but not celecoxib, delays the onset of sunitinib-induced hypertension

Absolute values of MAP and HR for each treatment group are displayed in Tables S1 and S2, respectively. The overall baseline mean arterial pressure (MAP) and heart rate (HR) were 114 ± 1 mmHg and 310 ± 2 bpm, respectively. Sunitinib induced a rapid increase in MAP, peaking in this group from 115 mmHg at baseline to 132 mmHg on day 4 (+17 ± 1 mmHg) and to 130 mmHg (+15 ± 2 mmHg) on day 6 (both $P < 0.001$ versus vehicle, respectively) (Fig. 1A). The sunitinib-induced increase in MAP was accompanied by a reduction in mean HR from 309 bpm to 281 p.m. (−28 ± 3 bpm) on day 2 ($P < 0.001$ versus vehicle) (Fig. 1B). The cumulative difference in MAP, expressed as area under the curve (AUC), was significantly higher after sunitinib compared to vehicle (AUC ΔMAP of +82 ± 6 mmHg versus +20 ± 6 mmHg respectively, $P < 0.001$) (Fig. 1C). The cumulative difference in HR was significantly lower in the sunitinib group compared to vehicle (AUC ΔHR of −114 ± 13 bpm versus 18 ± 12 bpm respectively, $P < 0.001$) (Fig. 1D).

Although co-treatment with celecoxib numerically blunted the maximal pressor response to sunitinib by approximately 30%, peaking from 115 mmHg to 127 mmHg (+12 ± 3 mmHg) on day 4 and to 126 mmHg (+11 ± 2 mmHg) on day 6, this difference did not reach statistical significance compared with sunitinib alone ($P = 0.247$ and $P = 0.167$, respectively) (Fig. 1A). Neither did co-treatment with celecoxib significantly lower the AUC ΔMAP compared with sunitinib alone (51 ± 17 versus 82 ± 6 mmHg respectively, $P = 0.103$) (Fig. 1C). Celecoxib significantly lowered the AUC ΔHR during the treatment period compared to sunitinib alone (−64 ± 14 versus −114 ± 13 bpm respectively, $P < 0.01$) (Fig. 1D).

During the first two days of treatment, co-treatment with iloprost significantly decreased the sunitinib-induced rise in MAP (−0.5 ± 2 mmHg versus +7.9 ± 1 mmHg on day 1, $P = 0.0075$, and 3.4 ± 2 versus +11 ± 2 mmHg on day 2, $P = 0.0125$) and prevented the decrease in HR (Fig. 1A + B). However, thereafter these effects did not persist and the maximal pressor response, peaking from 110 mmHg at baseline to 124 mmHg (+14 ± 1 mmHg) on day 4 and the overall AUC ΔMAP (55 ± 8 mmHg) did not differ significantly from the sunitinib alone treatment group ($P = 0.475$ and $P = 0.103$, respectively) (Fig. 1A + C). The AUC ΔHR (−28 ± 12 bpm) was significantly lower during co-treatment with iloprost ($P < 0.001$ compared with sunitinib alone) (Fig. 1D). Mean body weight was similar between groups at the end of treatment (Table S3).

3.2. Sunitinib does not increase circulating PGI_2 and this remained unaffected by celecoxib or iloprost

Treatment with sunitinib did not significantly affect circulating 6-keto-$PGF_{1\alpha}$ (Fig. 2A) and tended to decrease the TXB_2 levels ($P = 0.054$) (Fig. 2B). Consequently, sunitinib non-significantly increased the

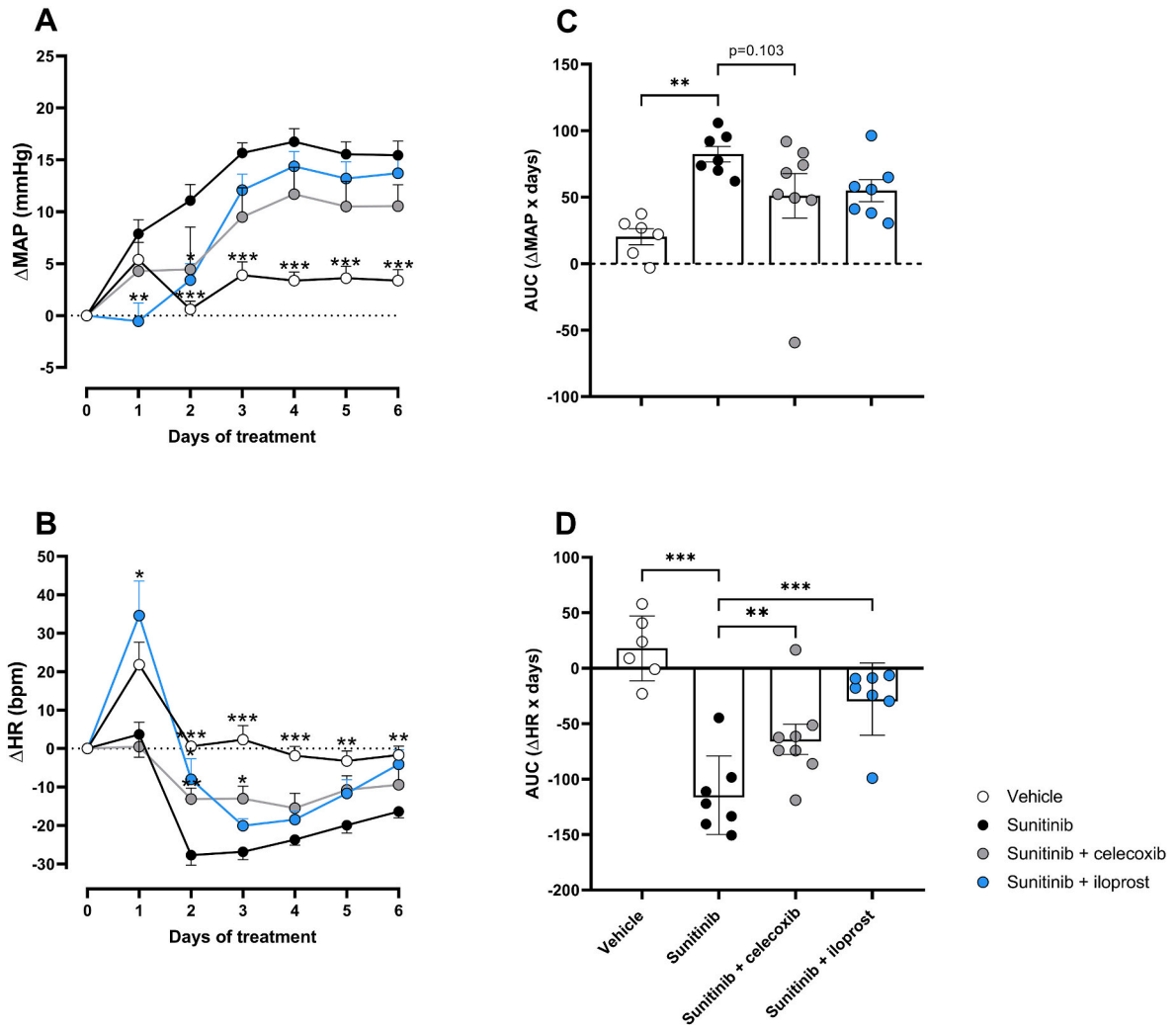

Fig. 1. Changes in mean arterial pressure (MAP) and heart rate (HR) in response to treatment with vehicle, sunitinib (14 mg/kg/day) alone or during co-treatment with celecoxib (selective COX-2 inhibitor, 10 mg/kg/day) or iloprost (prostacyclin analogue, 100 μg/kg/day). Time courses of changes in (**A**) MAP, (**B**) HR, with the cumulative change displayed by area under the curve (AUC) (**C**) and (**D**), respectively. Data are presented as mean ± SEM (n = 6–8 per group). ΔMAP and ΔHR were compared between groups by repeated measures ANOVA followed by Dunnett's multiple comparisons. AUC data were analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus sunitinib group.

circulating PGI_2/TXA_2 ratio by 5-fold versus vehicle (3.0 and 0.6, respectively, $P = 0.079$) (Fig. 2C) in line with previous data (Mirabito Colafella et al., 2022). Neither co-treatment with celecoxib nor with iloprost significantly altered circulating 6-keto-$PGF_{1\alpha}$, TXB_2 or their ratio (*PP*=═NS for all) (Fig. 2A–C). Circulating ET-1 levels were similar in all treatment groups (Fig. 2D). The increase in BP did not correlate with circulating levels of PGI_2 nor ET-1 (data not shown).

3.3. Celecoxib but not iloprost exerts renoprotection during sunitinib treatment

Sunitinib did not significantly increase plasma creatinine, which remained further unaffected by co-treatment with either celecoxib or iloprost (Fig. 3A). Sunitinib increased albuminuria (0.58 ± 0.05 versus 0.27 ± 0.04 mg/24h respectively, $P < 0.001$), and this was prevented by approximately 70% by co-treatment with celecoxib (0.36 ± 0.05 mg/24h, $P = 0.005$ versus sunitinib alone) but not iloprost (0.51 ± 0.04 mg/24h, $P = 0.317$ versus sunitinib alone) (Fig. 3B). No treatment altered 24-h urine volume, GFR or urinary creatinine excretion (Fig. 3C–E).

3.4. Sunitinib increases renal PGI_2 excretion without effects of celecoxib or iloprost

Sunitinib increased the renal excretion of 6-keto-$PGF_{1\alpha}$ by 20-fold ($P < 0.001$) (Fig. 4A) while the renal excretion of TXB_2 was unaltered ($P = 0.385$) (Fig. 4B). Consequently, the urinary PGI_2/TXA_2 ratio increased >25-fold during sunitinib administration ($P < 0.001$) (Fig. 4C). Co-treatment with celecoxib or iloprost did not affect these changes (Fig. 4A–C). Renal ET-1 excretion was similar in all treatment groups (Fig. 4D).

3.5. Sunitinib increases ET-1-mediated vasoconstriction, which remains unaffected by celecoxib and iloprost

In isolated iliac arteries of vehicle-treated rats, ET-1 induced vasoconstriction could be diminished by the ET_A receptor antagonist BQ123, but not the ET_B receptor antagonist BQ788 (Fig. 5A). Segments from sunitinib-treated rats demonstrated a significantly higher maximal vasoconstrictor response (Emax) to ET-1 compared with vehicle-treated rats (167% versus 105%, respectively, $P = 0.005$), which was dependent on the ET_A receptor (Fig. 5B). Prior *in vivo* treatment with celecoxib (Fig. 5C) nor iloprost (Fig. 5D) could reduce ET-1 mediated

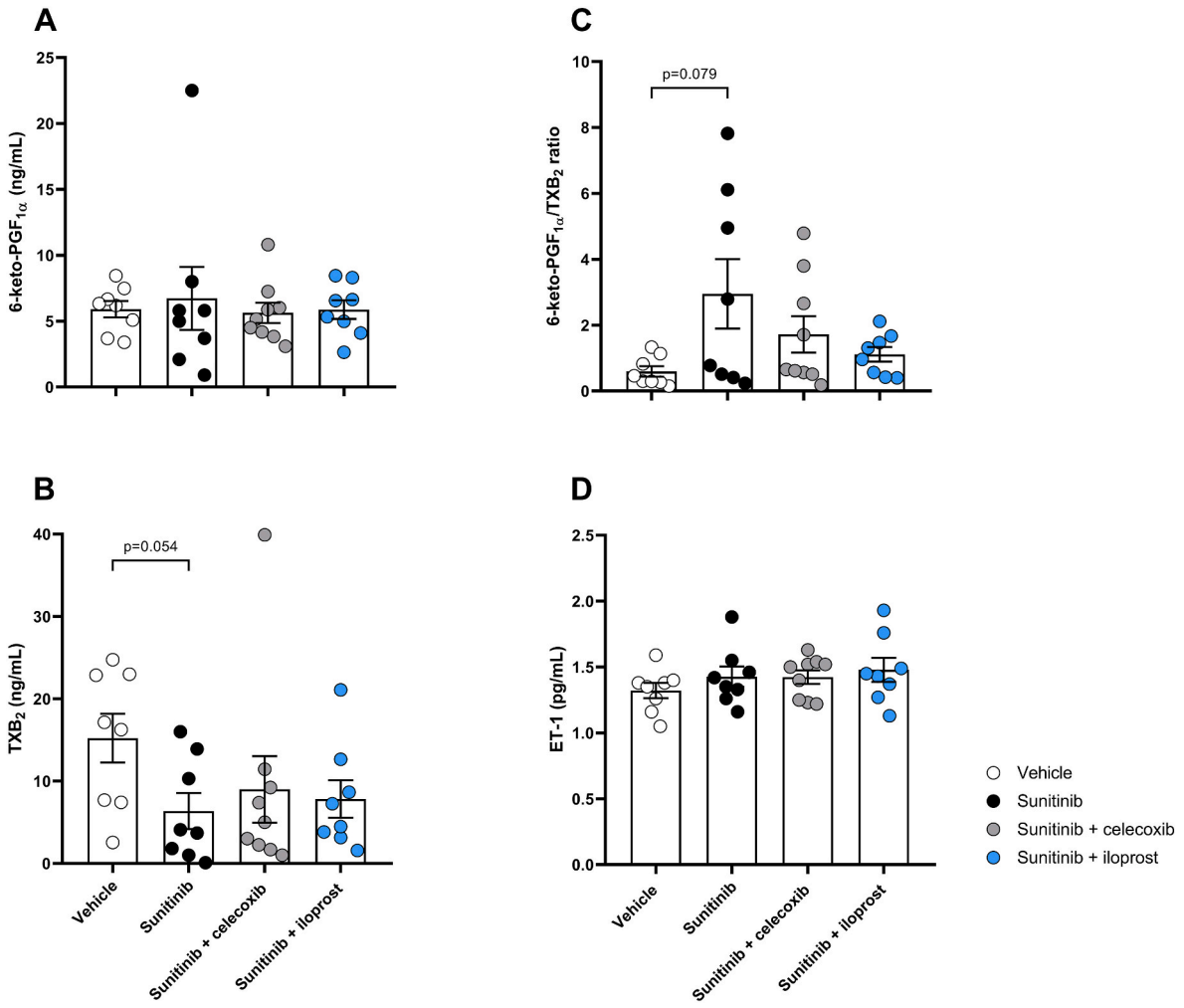

Fig. 2. (A) Circulating levels of prostacyclin (PGI_2, via its stable metabolite 6-keto-$PGF_{1\alpha}$), (B) circulating levels of thromboxane (TXA_2, via its stable metabolite TXB_2), (C) the 6-keto-$PGF_{1\alpha}$/TXB_2 ratio in the circulation, and (D) circulating levels of endothelin (ET)-1 following 8-day treatment with vehicle, sunitinib (14 mg/kg/day) alone or during co-treatment with celecoxib (selective COX-2 inhibitor, 10 mg/kg/day) or iloprost (PGI_2 analogue, 100 μg/kg/day). Data are presented as mean ± SEM (n = 8–9 per group). Levels of 6-keto-$PGF_{1\alpha}$, TXB_2 and the 6-keto-$PGF_{1\alpha}$/TXB_2 ratio were log-transformed before statistical analysis. Data were analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test.

vasoconstriction, evidenced by similar Emax and pEC50 [the negative logarithm of the half-maximal effective concentration] values. Under all circumstances, ET-1 mediated vasoconstriction remained dependent on predominantly ET_A receptor stimulation (Fig. 5A–D). Further statistical information can be found in Table S4.

3.6. PGI_2 exerts a modest degree of vasoconstriction via EP3 receptor stimulation

To study if PGI_2 would indeed be able to paradoxically induce vasoconstrictor effects, vasoconstriction response curves after *ex vivo* addition of iloprost were established in separate segments (Fig. 5E–H). In segments from vehicle-treated rats, iloprost exerted modest vasoconstrictor effects at high (micromolar) concentrations, which was unaltered following prior treatment with sunitinib alone (Fig. 5F) or co-treatment with celecoxib (Fig. 5G) or iloprost (Fig. 5H). EP3 receptor blockade with L-798106, but not TP receptor blockade with terutroban, tended to prevent this constrictor effect in all groups.

3.7. Sunitinib did not influence aortic nor renal COX-1 or COX-2 expression

Protein expression of both COX-1 and COX-2 was determined in aortic (Fig. 6A–C) and whole kidney (Fig. 6D–F) tissue. Sunitinib did not significantly affect expression of either COX-isoform in aorta ($P = 0.195$ and $P = 0.801$ compared to vehicle, respectively) (Fig. 6A–C) or kidney ($P = 0.999$ and $P = 0.877$ compared to vehicle, respectively) (Fig. 6D–F) and expression remained similar during co-treatment with celecoxib or iloprost (Fig. 6).

4. Discussion

Using an well-established rat model of sunitinib-induced toxicity (Lankhorst et al., 2015; Mirabito Colafella et al., 2020, 2022) we found that selective COX-2 inhibition could not significantly reduce the rise in MAP during sunitinib. Considering that high-dose aspirin in our previous study prevented the sunitinib-induced rise in MAP by approximately 50% (Mirabito Colafella et al., 2022), this suggests that dual COX-1 and COX-2 blockade, rather than selective COX-2 inhibition, is required to obtain the most efficient protection against hypertension during angiogenesis inhibition.

Celecoxib virtually normalized the effect of sunitinib on albuminuria without affecting urinary volume or GFR. This suggests that selective COX-2 inihbition can prevent renal injury during angiogenesis inhibition. High-dose apirin in our previous study (Mirabito Colafella et al., 2022) and celecoxib in the current study prevented sunitinib-induced

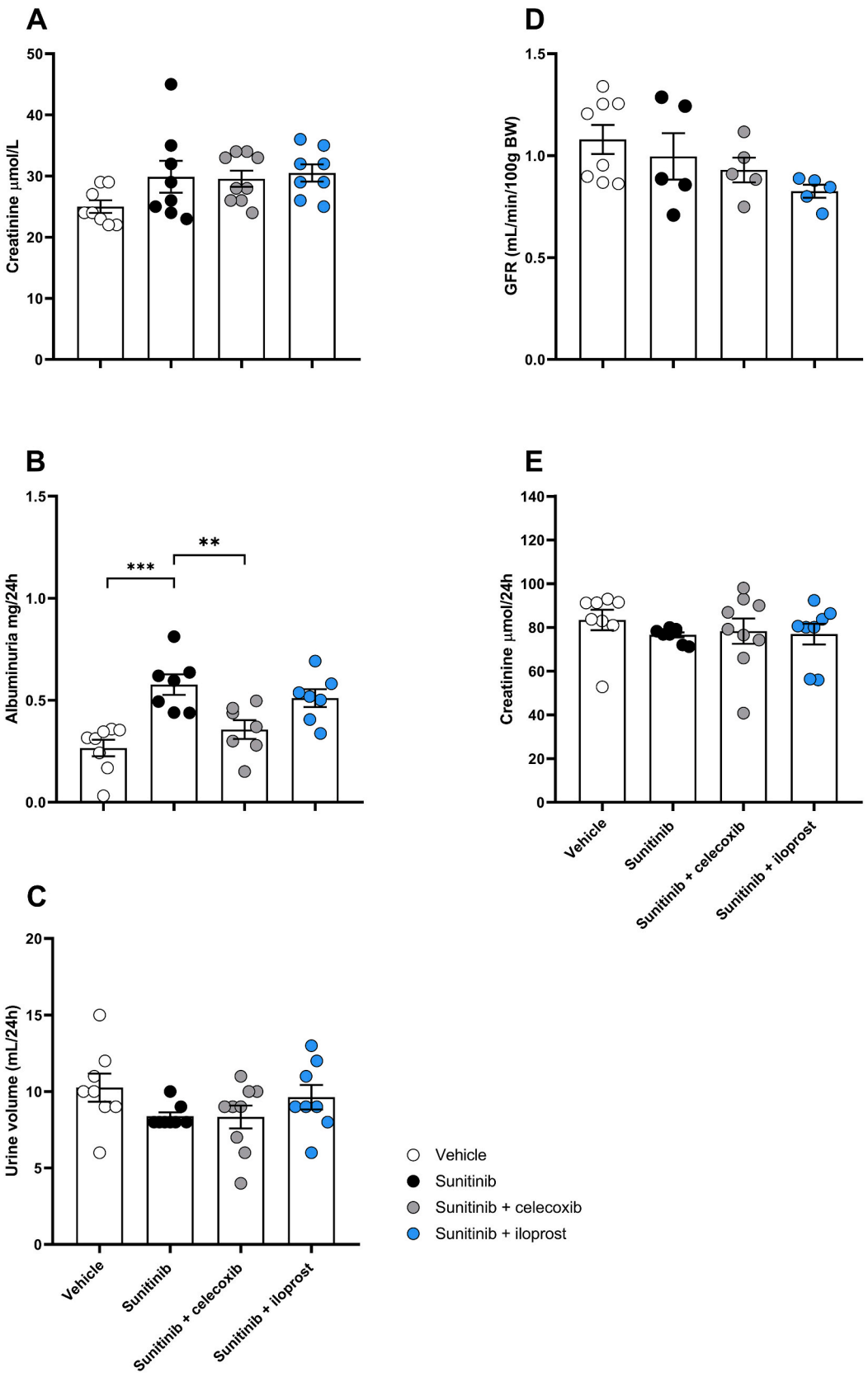

Fig. 3. Indices of kidney function and kidney damage: (**A**) plasma creatinine levels, (**B**) albuminuria, (**C**), 24h urine volume, (**D**) glomerular filtration rate (GFR), (**E**) urinary creatinine levels following 8-day treatment with vehicle, sunitinib (14 mg/kg/day) alone or during co-treatment with celecoxib (selective COX-2 inhibitor, 10 mg/kg/day) or iloprost (prostacyclin analogue, 100 µg/kg/day). Data are presented as mean ± SEM (n = 5–9 measurements per group). Data were analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$ versus sunitinib group.

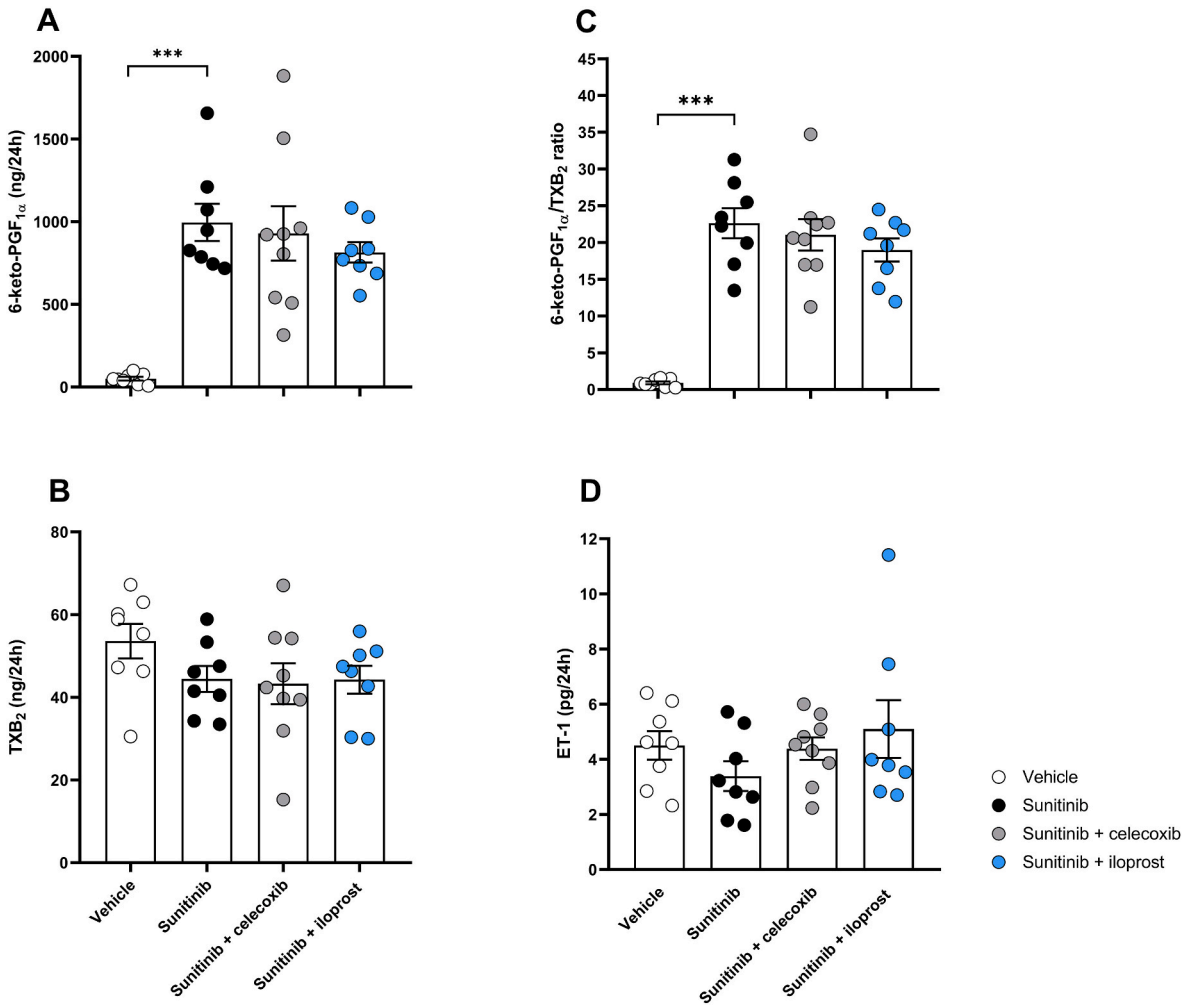

Fig. 4. (A) Urinary excretion of prostacyclin (PGI_2, via its stable metabolite 6-keto-$PGF_{1\alpha}$), (**B**) urinary excretion of thromboxane (TXA_2, via its stable metabolite TXB_2), (**C**) the urinary PGI_2/TXA_2 ratio, and (**D**) urinary excretion of endothelin (ET)-1 following 8-day treatment with vehicle, sunitinib (14 mg/kg/day) alone or during co-treatment with celecoxib (selective COX-2 inhibitor, 10 mg/kg/day) or iloprost (prostacyclin analogue, 100 μg/kg/day). Data are presented as mean ± SEM (n = 8–9 per group). Data were analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test. ***$P < 0.001$ versus sunitinib group.

albuminuria to the same extent (79% and 71% reduction, respectively). Therefore we identify COX-2 as a key contributor to the observed albuminuria and as a possible attractive strategy to mitigate renal toxicity during angiogenesis inhibition. The observation that celecoxib could only ameliorate sunitinib-induced albuminuria but not hypertension suggests that these side-effects are unrelated (Lankhorst et al., 2014).

It remains speculative how exactly COX-2 inhibition prevented sunitinib-induced albuminuria in the current study. One possibility is inhibition of ET-1-signalling by preventing COX-2 – ET-1 crosstalk (Lin et al., 2013; Zhou et al., 2006). It is already known that ET_A receptor antagonism prevents both the sunitinib-induced rise in blood pressure, and its renal toxicity (Mirabito Colafella et al., 2020), by interfering with ET-1-induced effects in the vascular wall and kidney, respectively. ET-1 is mainly released in an abluminal fashion (Stauffer et al., 2008) and this explains why its effects may even occur when no significant rises are seen in either blood or urine.

The beneficial effects of celecoxib on sunitinib-induced albuminuria could also be mediated via inhibition of COX-2-dependent prostanoid generation. Interestingly, in line with our current results, blood pressure-independent antiproteinuric effects of COX-2 inhibition have also been observed in patients with diabetic nephropathy or proteinuric nephropathy of glomerular origin (Vogt et al., 2008). The prostanoids PGE_2 and PGI_2 are the most logical candidates, given preferential coupling of COX-2 to PGE_2 - and PGI_2 synthase (Caughey et al., 2001). In addition, renal production of PGE_2 and PGI_2 is upregulated to protect against renal injury and to maintain renal blood flow, particularly during conditions of decreased perfusion (Kim, 2008). The latter is likely to occur during sunitinib-induced glomerular ischemia (Lankhorst et al., 2015). Nevertheless, sunitinib upregulated only renal PGI_2 but not PGE_2 excretion (Mirabito Colafella et al., 2022), and celecoxib did not alter this outcome, nor did iloprost further aggravate the sunitinib-induced renal toxicity. These data suggest that factors other than PGI_2 are responsible for albuminuria during angiogenesis inhibition, and that the rise in PGI_2 during angiogenesis inhibition likely constitutes a renoprotective mechanism. Indeed, albeit to a lesser extent than PGE_2, PGI_2 has been shown to increase natriuresis (Kim, 2008; Villa et al., 1997), and renal PGI_2 upregulation could thus be a physiological attempt to combat the salt sensitivity of the blood pressure response (Lankhorst et al., 2016; van Doorn et al., 2023) during angiogenesis inhibition. Given that sunitinib in the current dose (14 m/kg/day) previously did not cause gross histological abnormalities nor a significant increase in kidney injury markers KIM-1 and NGAL (Lankhorst et al., 2015; Mirabito Colafella et al., 2022), these parameters were not determined again in the current study.

Addition of a PGI_2 analogue attenuated, rather than worsened, the sunitinib-induced vasopressor response during the first days of treatment. This argues against the concept that PGI_2 is responsible for the rise

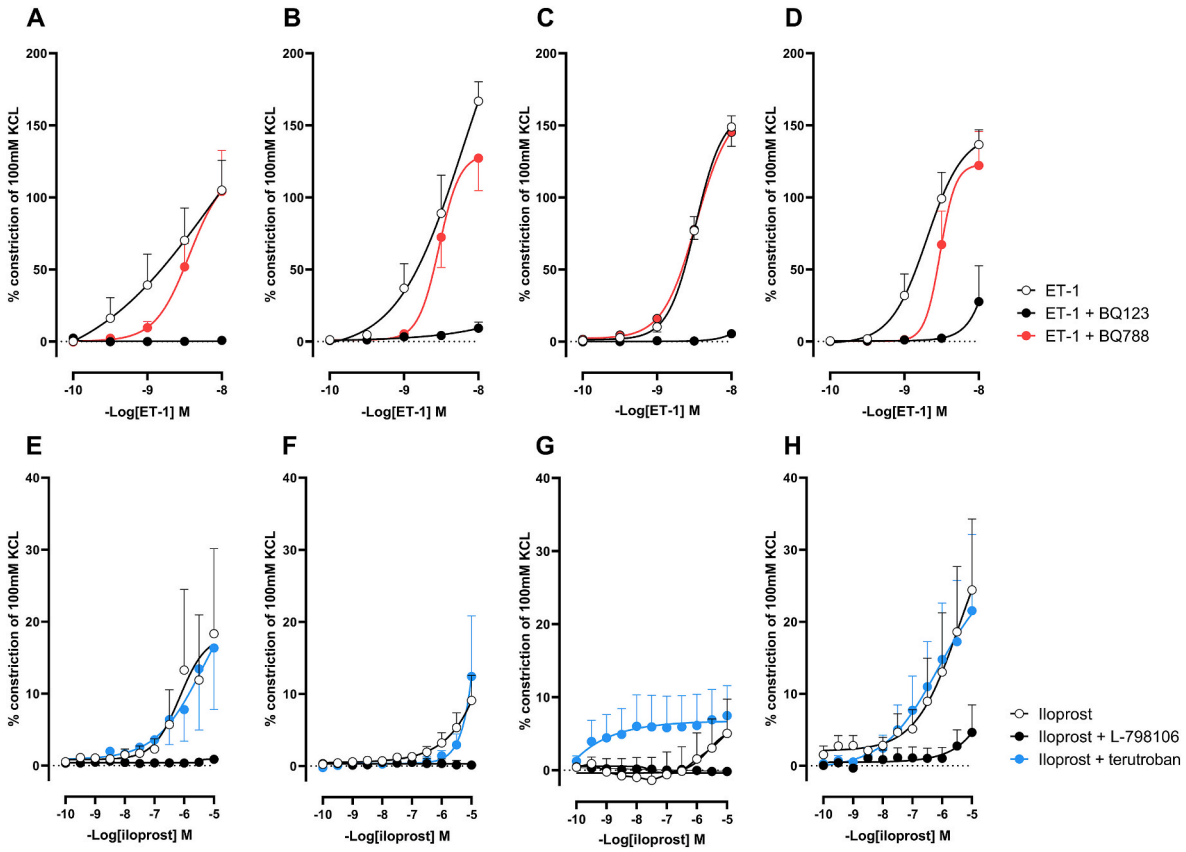

Fig. 5. Iliac artery concentration-response curves to (**A-D**) endothelin (ET)-1 in the absence or presence of BQ123 (ET_A receptor antagonist, 10^{-6}M) or BQ788 (ET_B receptor antagonist, 10^{-6}M) or to (**E-H**) iloprost in the absence or presence of L-798106 (EP3 receptor antagonist, 10^{-6}M) or terutroban (TP receptor antagonist, 10^{-6}M) following 8-day *in vivo* treatment with vehicle, sunitinib ((SU) 14 mg/kg/day) alone or during co-treatment with celecoxib (selective COX-2 inhibitor, 10 mg/kg/day) or iloprost (prostacyclin analogue, 100 μg/kg/day). Data are presented as mean ± SEM (n = 4–7 for every *in vivo* treatment group). See Table S4 for statistical information.

in blood pressure during sunitinib, and rather suggests that PGI_2 upregulation is a compensatory mechanism. While assessing PGI_2 levels, the ratio between PGI_2 and TXA_2 has to be taken into account because these prostanoids are normally delicately balanced due to their opposing effects on the vasculature (Atallah et al., 2017; Mirabito Colafella et al., 2019). None of the co-treatments significantly altered these prostanoids nor their ratio compared with sunitinib alone. Of note, PGI_2 itself has been reported to act as an endothelium-derived contracting factor via stimulation of prostanoid receptors other than its cognate IP receptor, particularly under pathological conditions and in excessive concentrations (Vanhoutte, 2011; Vanhoutte and Tang, 2008). In the present study, we did observe such PGI_2-induced constrictor effects in iliac arteries, which appeared to rely on EP3 receptor stimulation. However, the effects were small and required high (micromolar) PGI_2 concentrations. PGI_2 rises in blood during angiogenesis inhibition in previous studies in humans and rats (based on its stable metabolite 6-keto-$PGF_{1\alpha}$) were maximally around 2-fold, implying that the levels in blood were in the nanomolar range, i.e. far below the levels needed to stimulate EP3 receptors. In addition, PGI_2-induced vasoconstriction was not facilitated by previous sunitinib treatment. It is important to note that, to the best of our knowledge, the PGI_2 analogue iloprost is not metabolized into 6-keto-$PGF_{1\alpha}$ in rodents (Hildebrand, 1992; Krause et al., 1984). This could explain why iloprost treatment did not upregulate circulating nor urinary 6-keto-$PGF_{1\alpha}$ levels. In summary, 1) sunitinib-induced hypertension was not aggravated by a PGI_2 analogue, 2) sunitinib did not increase circulating PGI_2 levels whilst still exerting prohypertensive effects, and 3) sunitinib did not increase PGI_2-mediated vasoconstriction. This argues against PGI_2 as a major contributing factor to sunitinib-induced hypertension, and at most supports a short-term compensatory role of PGI_2 counteracting the hypertensive effect of sunitinib. These data likely indicate that the blood pressure-lowering effect of dual COX-1 and COX-2 blockade is independent of PGI_2 generation. Collectively, our results suggest that selective COX-2 inhibition is an attractive target to ameliorate renal injury during angiogenesis inhibition. The beneficial effects of COX-2 blockade are not predominantly mediated by, and are most likely independent of, PGI_2. To combat angiogenesis inhibitor-induced hypertension, dual COX-1 and COX-2 blockade, rather than selective COX-2 blockade, seems preferential.

The current study has some limitations. Despite the use of a well-established animal model for sunitinib-induced toxicity (Lankhorst et al., 2015; Mirabito Colafella et al., 2020, 2022), we only studied the effects of a single treatment dosage of celecoxib and iloprost. Although the administered doses of celecoxib and iloprost were carefully based on previous studies (Amraoui et al., 2014; Elmarakby et al., 2018; Helmy et al., 2019; Koçkaya et al., 2010; Muscará et al., 2000; Xie et al., 2019; Zlatnik et al., 1999) we cannot rule out that higher doses of these drugs might have yielded different outcomes. Moreover, we collected plasma samples after 8 days of treatment only, and it is possible that the changes in PGI_2, ET-1, and TXA_2 were more prominent at an earlier time point. This is particularly true for PGI_2, given that the PGI_2 analogue tended to prevent sunitinib-induced hypertension in the initial stage only. Future studies should additionally measure circulating PGI_2 levels at these earlier timepoints in order to acquire more insights in the possible (protective) role of PGI_2 during the first days of angiogenesis inhibition treatment. In addition, we measured 6-keto-$PGF_{1\alpha}$ as the main PGI_2 metabolite in our study, whereas more metabolites derived from PGI_2 and other prostanoids could have contributed to angiogenesis inhibitor-induced toxicity. Nonetheless, 6-keto-$PGF_{1\alpha}$ is a frequently

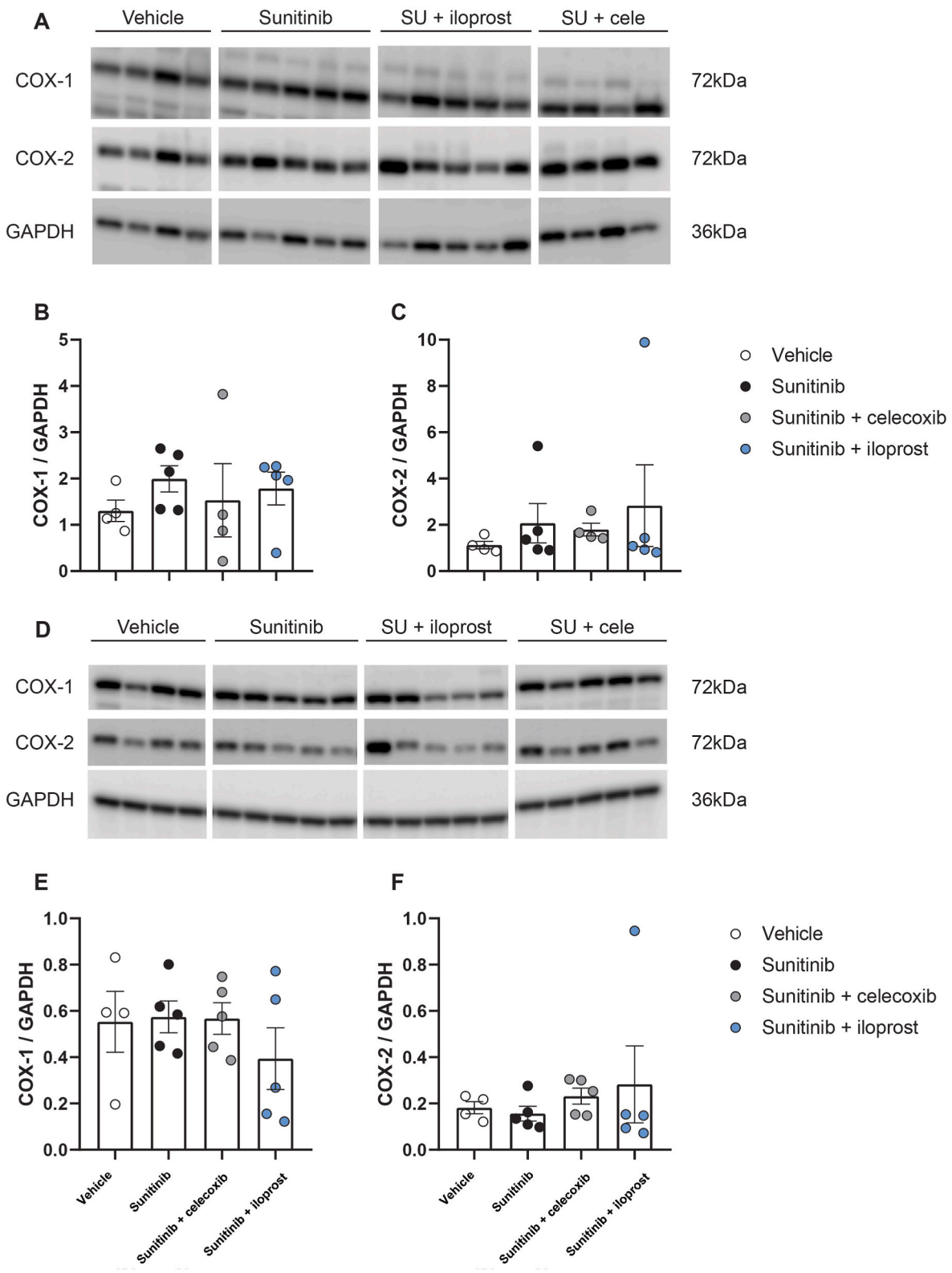

Fig. 6. Aortic (**A**) western blots and quantification of (**B**) COX-1 and (**C**) COX-2 expression relative to GAPDH, and kidney (**D**) western blots and quantification of (**E**) COX-1 and (**F**) COX-2 expression relative to GAPDH following 8-day treatment with vehicle, sunitinib (14 mg/kg/day) alone or during co-treatment with celecoxib (selective COX-2 inhibitor, 10 mg/kg/day) or iloprost (prostacyclin analogue, 100 μg/kg/day). Data are presented as mean ± SEM (n = 4–5 per group). Samples from all treatment groups were loaded on the same gel and measured in parallel. Samples for quantification of COX-1 and COX-2 expression were loaded on separate gels. GAPDH was determined on the same gel as COX-1. Aortic and renal expression of COX-2 was log-transformed before statistical analysis. Data were analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test. Abbreviations: cele = celecoxib, SU = sunitinib.

measured stable PGI_2 metabolite which is produced in vascular tissue as well (Gluais et al., 2006; Kent et al., 1983). In our previous study in the same animal model only PGI_2, and not PGE_2, nor PGE_2-M or 8-iso-$PGF_{2\alpha}$ levels were affected by sunitinib (Mirabito Colafella et al., 2022). This is why we specifically focused on PGI_2 here. Lastly, the *ex vivo* vascular reactivity and contribution of various vascular receptors during the wire myography experiments was assumed to be reflective of the situation *in vivo*.

Future studies are required to further investigate the COX1/2-selectivity and the roles of the ET-1 – COX-2 crosstalk, and PGI_2 and other COX-derived prostanoids in angiogenesis inhibitor-induced toxicity. Additionally, it would be of great interest to study the contribution of the different prostanoid receptors, e.g., the EP3 receptor given its involvement in salt-sensitive hypertension and renal inflammation (Wu et al., 2021; Xiao et al., 2019). In this context, administration of a PGI_2 synthase antagonist together with sunitinib and/or an EP3 receptor antagonist would be an interesting option to further investigate the exact role of PGI_2. Of note, a complete PGI_2 synthase knockout animal model seems suboptimal given its inherent associations with hypertension and severe renal abnormalities (Yokoyama et al., 2002). Eventually, further delineation of the role of COX-derived prostanoids and their receptors might lead to the identification and development of novel therapeutic strategies to combat angiogenesis inhibitor-induced toxicity in patients with cancer.

Funding

Not applicable.

CRediT authorship contribution statement

Daan C.H. van Dorst: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Project administration, Writing – review & editing. **Katrina M. Mirabito Colafella:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Richard van Veghel:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Ingrid M. Garrelds:** Data curation, Methodology, Writing – review & editing. **René de Vries:** Data curation, Methodology, Writing – review & editing. **Ron H.J. Mathijssen:** Conceptualization, Supervision, Funding acquisition, Methodology, Project administration, Writing – review & editing. **A.H. Jan Danser:** Conceptualization, Supervision, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Jorie Versmissen:** Conceptualization, Supervision, Funding acquisition, Methodology, Project administration, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2023.176199.

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