Tetradecylthioacetic acid down-regulates cyclooxygenase 2 in the renal cortex of two-kidney, one-clip hypertensive rats

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Running head: TTA and COX2 in renal hypertension

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ABSTRACT
The effect of tetradecylthioacetic acid (TTA) on the cyclooxygenase (COX) system was investigated in 2K1C hypertensive rats. The systolic blood pressure (BP) was increased 6 weeks after clipping to 183 ± 4 mmHg vs. 127 ± 3 mmHg in TTA treated 2K1C rats. The COX1 protein expression was not affected either by the 2K1C procedure or by TTA treatment. COX2 expression was up-regulated in both kidneys, but to a greater extent in the clipped kidney. COX2 activity was 16 ± 3% in control and 38 ± 2% (p<0.001) in the clipped kidney, and COX2 protein expression was 1.3 ± 0.04 in control and 1.6 ± 0.12 in the clipped kidney (p=0.006). TTA reduced COX2 activity to control levels. Subcutaneously infusion of a COX2 inhibitor did not reduce BP. Peroxisome proliferator-activated receptors (PPARs) were detected in both kidneys and PPAR delta was up-regulated in the non-clipped kidney after TTA treatment. Prostaglandin E$_2$ (PGE$_2$) in renal cortex was increased in 2K1C (31 ± 0.3 in the clipped and 28 ± 0.2 pg ml$^{-1}$ non-clipped kidney, p<0.001 compared to control). TTA lowered the PGE$_2$ to control level. Renal blood flow (RBF) response to exogenous angiotensin II (ANG II) injected into the control and non-clipped kidney was exaggerated after indomethacin treatment, but unchanged in the non-clipped kidney of 2K1C TTA group. Overall, these results indicate that after 6 weeks of treatment, TTA down-regulated the COX2 activity, which have potentially important effects on the regulation of renal hemodynamics but does not explain TTAs ability to lower blood pressure.

Key words: Cyclooxygenase, RBF, ANG II, PGE$_2$
INTRODUCTION

Two kidney, one clip (2K1C) is a model of renal hypertension where the renin-angiotensin system is highly activated (28, 32). The renin level in the clipped kidney is increased in contrast with what is found in the non-clipped kidney where the renin level is low (10, 23). On the other hand, angiotensin II (ANG II) concentration is enhanced in both kidneys (10), indicating that different mechanisms of ANG II regulation is present in the clipped and non-clipped kidney (23, 32). While the renin production in the clipped kidney is the major driving force behind the hypertension, the non-clipped kidney also seems to play an important role in the maintenance of hypertension (40). The high level of ANG II in this kidney seems to depend on admission of ANGII from plasma via an angiotensin II type 1 receptor (AT1R) dependent pathway (1, 31). Decreased AT1R density has been reported in both clipped and non-clipped kidney 4 weeks after clipping (20), due to internalization, desensitization or phosphorylation of AT1R via either ANG II dependent or independent pathways (17, 34). Renal blood flow response (RBF) to exogenous ANG II has been reported to be decreased in the non-clipped kidney (8).

Prostaglandins (PG) are involved in regulation of many processes in the human body (26, 47), and PGE$_2$, a member of PG family, is involved in hypertension, inflammation (47) and, importantly, the regulation of kidney function (43, 47). The key enzymes in the generation of prostaglandins are the cyclooxygenases (COX) (47). Both COX1 and COX2 are constitutively present in the kidney cortex (19, 48, 53). In addition COX2 is induced in 2K1C renovascular hypertension (19, 27) as well as in other models with high ANG II levels (24). The PG-E synthases (PGES) are downstream enzymes that produce PGE$_2$, and COX2 may be more selectively linked to the terminal PGE$_2$ synthase than COX1 (30, 45). Increased PGE$_2$
production may thus indicate increased COX2 activity (36, 47, 57). Indeed COX2 selective blockers reduce the levels of PGE$_2$ and inflammatory response (29, 55).

It has been shown that COX2 and renin expression have a parallel regulation (19, 21) and selective COX2 blockers have been shown to reduce renin activity (19). For example, in COX2 deficient mice, the plasma renin level is low with reduced response to acute stimulation (25). In addition, there are studies that have shown reduction of blood pressure after treatment with COX2 blockers in both 2K1C hypertension and inducible ANG II-dependent malignant hypertension in CYP1A-Ren2 transgenic rats (35, 56), although other reports show no effect on blood pressure after COX2 inhibition as shown in a recent study by Richter et al. (41).

Tetradecylthioacetic acid (TTA) is a modified fatty acid that can not undergo β-oxidation (2), and it is known to exerts its various effects (4) by peroxisome proliferator-activated receptor (PPAR) dependent or independent pathways (3, 14). We have recently shown that TTA prevents the development of hypertension in 2K1C by reducing the activation of the renin-angiotensin system (5, 16) and furthermore, that TTA has a strong anti-inflammatory effect (6). TTA reduces mRNA renin levels in the clipped kidney and ANG II in both clipped and non-clipped kidney (16). As COX2 is enhanced in high renin models and TTA reduces the activity of the renin-angiotensin system, we hypothesized that TTA would decrease COX2 activity and PGE$_2$ production, mechanisms that may contribute to blood pressure lowering effect in 2K1C hypertension.

**MATERIALS AND METHODS**

*Animals.* The study was performed in 60 Wistar male rats Han.Tac: WH M from Møllegaard Breeding Colony (Skensved, Denmark) with a body weight of 180-200 g at the
beginning of the study. The experiments were performed in accordance with, and under the approval of the Norwegian State Board for Biological Experiments, the Guide for the Care and Use of Laboratory Animals and the Guidelines of the Animal Welfare Act. The rats were hosted in cages with constant temperature (25ºC) and humidity. They were exposed to a 12:12 h light-dark cycle (light from 6:00 pm to 18:00 am) and had unrestricted access to water and food.

**Induction of two-kidney-one clip (2K-1C) hypertension.** The right kidney was exposed through a lumbar incision and the right renal artery was dissected free and clipped by a rigid U shaped silver clip with an internal opening of 0.25 mm. The animals were anesthetized by isoflurane anesthesia 1l/min (Forene – Abbott) mixed with 2L/ min O2 using an Ohmeda Isotec 3 anesthesia utility (BOC–Health Care). To reduce the pain following the surgery, the rats received Temgesic 1ml kbw⁻¹ day⁻¹ after the surgery and in the next 2 days. The animals were followed for the next 6 weeks until the renal hemodynamic studies were done, or the collection of plasma, urine and kidney were made.

**Measurements of blood pressure.** Systolic blood pressure (BP) was measured before clipping and weekly during the development of hypertension by means of the tail-cuff method (UGO BASILE) in unanaesthetized animals. The rats were pre-warmed to 35ºC for 10 min in a cupboard before the measurements.

**The study groups.** The animals were randomized in four sets of experiments, each set consisting of three groups: one non-clipped control group and two groups with 2K1C hypertension, whereof one group was non-treated (abbreviated as 2K1C) and one group was TTA-treated (abbreviated as 2K1C TTA). The first set of experiments was used for collection
of renal cortex and urine (N = 6 in each group). As TTA do not affect BP in control rats this
group was not examined in the present study (5, 6). One urine sample per rat was collected as
well as one tissue sample from the left kidney in the control group and one sample from each
kidney in the 2K1C group. The second set of experiments was used for renal hemodynamic
study (N = 6 in each group). The third group was used for studies of PPARs in clipped and
non-clipped kidneys with and without TTA treatment. Furthermore, one group was used to
study the effect of a COX2 inhibitor on blood pressure The control and 2K1C hypertensive
groups were fed with standard rat chow (0.25 % Na, 14.7 % proteins) and the TTA group was
fed standard rat chow that was sprayed with TTA dissolved in acetone (9g TTA dissolved in
1L acetone to 2.7 kg pellet). We have been shown previously that TTA and acetone did not
interfere with the food intake (5, 16).

Collection of urine. The urine samples were collected from unanaesthetised rats six
weeks after clipping and start of TTA-treatment. The urine was collected on ice for 24h using
metabolic cages. The urine volume was recorded as ml per h, and 1 ml of each sample was
filtered and frozen at -80ºC until analysed.

Collection of renal cortex. The kidneys were collected from anesthetized rats and
immediately frozen in liquid nitrogen. When homogenised, the kidneys were kept for 30s at
room temperature and the cortex was isolated, weighted and homogenised in lysis buffer (5ml
per g tissue, TRIS-HCl 10mM, pH 7.4 with 10µM EDTA, 1.5 mM MgCl₂, 10 mM KCl, 0.1%
Triton X-100 and EDTA-free proteinase inhibitor mixture-Roche). The homogenised samples
were centrifuged at 10000g for 15 min at 4°C and the supernatant was kept at -80ºC until
analysed.
Measurements of cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2) activity in renal cortex. COX1 and COX2 activity was measured using a kit for the peroxidase activity of cyclooxygenase (Cayman Chemical, No. 760151). The peroxidase activity was assayed colorimetrically by monitoring the appearance of oxidised N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), a stable metabolite of PGH\textsubscript{2}, in the presence of arachidonic acid. When assayed, the samples were treated with SC-560 (COX1 selective inhibitor), and DuP-697 (COX2 selective inhibitor). Inactive samples, obtained by placing these in boiled water 5 min, were assayed as backgrounds for each sample. Arachidonic acid was added and the oxidised PGH\textsubscript{2} was quantified colorimetrically at 590 nm. The reaction rate was determined using the TMPD extinction coefficient (0.00826 µM\textsuperscript{-1}). One unit was defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of TMPD per min. at 25 °C. The total COX activity for each sample was calculated in nmol min\textsuperscript{-1} ml\textsuperscript{-1} (U ml\textsuperscript{-1}). The COX1 or COX2 activity in each sample was calculated as percentage from total COX activity.

Western Blot for COX1 and COX2 from kidney cortex. Homogenates of kidney cortex (1g tissue per 5 ml buffer) were prepared in 25mM Tris-HCl -pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1mM PMSF with proteinase inhibitor mixture (Roche). The homogenate was centrifuged and the resulting supernatant was collected as cytosolic fraction. The COX1 and COX2 expressions were assessed by standard SDS-PAGE Western blotting techniques. The proteins were separated by electrophoresis on a 10% SDS-PAGE with TRIS glycine buffer (10% SDS, 24 mM Trisma base, 192 mM glycine) for 1h at 150V. The transfer was made for 1h at 100V on a polyvinylidene difluoride transfer membrane (Amersham). The membrane was blocked by skimmed milk in TRIS buffered salt solution (TBS: 19mM TRIS, 0.5 mM NaCl) for 1h at room temperature and washed with TBS-Tween (TBS with 0.05% Tween 20). The membrane was then incubated with mouse monoclonal anti-COX1 antibody (11 SC-
19998) or mouse monoclonal anti-COX2 antibody (29 SC-19999, Santa Cruz Biotechnology) for 1h at room temperature. After washing (3 x 10 min with TBS-Tween at room temperature) the membrane was incubated with secondary antibody (SC-2005, Santa Cruz Biotechnology).

The protein marker was a dual colour one from Bio-Rad (cat no. 161. 1374). Enhanced chemiluminescence detection was used (ECL from Amersham). The membranes were developed by a Bio-Rad Laboratory utility. Actin was used as loading reference and Western blot detection was done by the same protocol as for COX1 and COX2. Antibodies used were: mouse monoclonal (4i374 SC-70318) and secondary antibody (SC-2005, Santa Cruz Biotechnology). Densitometric analyzes of the blots were done by Image J software and the ratio COX1/actin and COX2/actin was calculated.

*Measurement of PGE$_2$ in kidney cortex and urine.* The PGE$_2$ was measured by an enzymatic immunoassay kit from Cayman Chemical (cat. No 514531.1). The assay was based on the competition between the stable metabolite of PGE$_2$ (PGEM) and PGEM-acetylcholinesterase conjugate as tracer against a limited number of PGEM specific rabbit antiserum binding sites. The unbound reagents were removed by wash and the substrate for the acetylcholinesterase (Elmans reagent) was added. The absorbance was recorded with a 412nm filter.

*Real-time quantitative RT-PCR for PPARs* Total RNA was purified from frozen kidneys using RNeasy Midi Kit (Qiagen, Hilden, Germany). PPAR$\alpha$, PPAR$\delta$ and PPAR$\gamma$ are “Assay-on Demand genes” designed by Applied Biosystems (CA, USA), with the following assay ID numbers: Rn00566193_m1 (PPAR$\alpha$), Rn00565707_m1 (PPAR$\delta$) and Rn00440945_m1 (PPAR$\gamma$). Real-time PCR was carried out in
triplicate for each sample on an ABI 7900 sequence detection system (Applied Biosystems). A dilution curve from one cDNA source using dilutions 1:2, 1:4, 1:8 and a no-template control was run for each gene. The gene expression was determined by relative quantification using the standard curve method. For each sample, results were normalized to 18S rRNA (RT-CKFT-18S, MedProbe) by comparing changes in threshold cycles.

**Hemodynamic study.** The left kidney from control rats and the left non-clipped kidney from 2K1C and TTA-treated rats were used for measurements of renal blood flow (RBF) in response to ANG II injections in the renal artery 6 weeks after clipping. The ANG II concentration (2.5 ng) used did not change the systemic blood pressure recorded as mean arterial pressure (MAP). The experiments have been described before (bivol et al) REF HER!. In short, anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (65 mg·kg⁻¹·kg BW) and the animals rats were placed on a servo-controlled heating table to keep the body temperature at 38.0 ± 0.5 °C. Tracheotomy was performed to facilitate free breathing, and the animals received oxygen during the acute experiment.

The right femoral artery was cannulated with a PE-25 catheter to monitor arterial pressure using a Statham P 23 XL pressure transducer. Another PE-25 catheter was introduced into the right femoral vein for the infusion of albumin, injection of indomethacin and supplementary doses of pentobarbital sodium. Bovine serum albumin (BSA, 4.7 g·dl⁻¹) was dissolved in isotonic saline containing Heparin heparin (0.15 ml per 10 ml saline). The BSA solution was infused initially at a rate of 50 µl·min⁻¹ to replace losses associated with surgery (1.25 ml·100g⁻¹·kg BW⁻¹), and then at 10 µl·min⁻¹ during the experiment.
The abdominal aorta and left kidney were exposed through a midline incision. For injection of ANG II, a PE-10 catheter was introduced into internal iliac artery and then advanced until its tip was positioned approximately 2-3 mm inside the left renal artery without affecting the renal blood flow. The left renal artery was localized and dissected free. The adjacent fat tissue was carefully removed for proper acoustical coupling. An ultrasound V Transonic System Inc. flow probe with an internal diameter of 0.8 mm, was placed on the renal artery. The Dane-Gel E2 (Rohde Produits) aqueous–transmission was used as an acoustical couplant. The flow probe was interfaced to the data acquisition system using a Transonic T 206 electromagnetic flowmeter and a Gould TA 5000 recorder installed on a PC compatible computer with a 12-bit analogue to digital converter. A Gould TA 5000 Hewlett Packard carrier amplifier was used for the pressure transducer sensor interface. The outputs of the transducer monitoring arterial pressure and RBF were sampled at a rate of 1 sample s\(^{-1}\). Each recording started 15 sec before injection of vasoconstrictor (ANG II) in left renal artery. RBF values were measured in ml min\(^{-1}\) and expressed as a percent of baseline values.

After completion of the surgical preparation, the animals were allowed to stabilize for 1 h before starting the records of MAP and RBF response to ANG II injections in left renal artery. Initially, the renal vascular response to 2.5 ng ANG II was recorded as basic line vascular response. Thereafter indomethacin (1ml kg\(^{-1}\) kg bw) was infused through the right venous femoral catheter. Indomethacin was prepared by diluting 5 mg indomethacin in 1 ml distilled water, buffered by 5 mg sodium carbonate. Injections of ANG II were performed 20 min after indomethacin treatment and RBF response to 2.5 ng ANG II was recorded as vascular response. At the end of experiments, the rats were sacrificed by an extra dose of pentobarbital. The kidneys were removed and weighed.
Infusion of a COX 2 inhibitor: A separate group of 12 animals was prepared for 2K1C as described above and subcutaneously infused with the specific COX2 inhibitor parecoxib (5 mg/kg/24h, Pfizer, NY) using osmotic minipumps (Alzet, CA, 2ML4) for 4 weeks after surgery. Blood pressure was investigated at the end of treatment using the tail-cuff method described above.

Chemicals: ANG II, bovine serum albumin and indomethacin were acquired from Sigma-Aldrich. Heparin (100 IE ml⁻¹) was bought from Leo-Pharma.

Statistical methods. Data are reported as mean ± standard error. Statistical analyses were done using SPSS for Windows 2003. Differences between groups were assessed by one way analysis of variance (ANOVA), followed by post-hoc Scheffé correction. For RBF measurements before and after indomethacin, a p-paired Student t-test was used to assess the differences within the same group. P < 0.05 were considered statistically significant.

RESULTS

Systolic blood pressure (BP). The BP was not significantly different between the groups before clipping, and the BP in the control group was unchanged after 6 weeks (108 ± 3 mmHg). In the 2K1C hypertensive group, the BP was significantly increased to 183 ± 4 mmHg 6 weeks after clipping when compared to 110 ± 2 mmHg before clipping (p<0.001) and to 108 ± 3 mmHg in controls (p<0.001). In the 2K1C TTA-treated group, BP was significantly lower 6 weeks after clipping when compared to 2K1C (128 ± 3 mmHg, p<0.001), but the BP remained higher than before clipping (p<0.001) (Fig.1).
**COX1 activity in renal cortex.** COX1 activity was calculated as % of the total COX activity. In the kidney cortex from the control group, the COX1 activity was 80 ± 3%. In the clipped kidney from 2K1C rats, the COX1 activity was reduced to 61 ± 4% (p=0.004 compared to control), while in the TTA treated 2K1C group, COX1 activity was similar to control values (83 ± 3%), and significantly higher than the 2K1C group (p=0.001) (Fig.2A). In the non-clipped kidney of the 2K1C group, the COX1 activity was 66 ± 3% (p=0.009 compared to control). After TTA treatment in the 2K1C group, the COX1 activity in this kidney was similar to control (76 ± 2%), and higher when compared to 2K1C group (p=0.02) (Fig.2C).

**COX2 activity in renal cortex.** The COX2 activity was calculated as % of the total COX activity. The COX2 activity in the renal cortex from the control was 16 ± 3%. In the clipped kidney of 2K1C group, COX2 activity was nearly 2 times higher (38 ± 2%) compared to controls (p<0.001), and after TTA treatment the COX2 activity in this kidney was similar to controls (18 ± 2%), but lower when compared to 2K1C group (p<0.001) (Fig. 3C). In the non-clipped kidney of 2K1C, the COX2 activity was lower than in the clipped kidney, but still significantly higher then controls (26 ± 2%, p=0.02). In the TTA treated 2K1C group, COX2 activity in the non-clipped kidney was 22 ± 2%, similar to control value, but lower when compared to 2K1C group (p=0.04) (Fig.3C).

**Protein expression for COX1.** COX1 was detected at 70 kd. There was no difference in COX1 protein expression between the groups. The ratio COX1/actin was 1.6 ± 0.08 in control group, while in 2K1C group was 1.3 ± 0.11 in the clipped kidney and 1.2 ± 0.09 in the non-clipped kidney. After TTA treatment, the ratio COX1/actin was 1.4 ± 0.12 in the clipped kidney and 1.6 ± 0.09 in the non-clipped kidney of TTA treated group (fig.2A). There was no difference between COX1 protein expression in 2K1C and 2K1C TTA treated group in the
clipped kidney, but TTA treatment increased the COX1 protein level compared to 2K1C in the non-clipped kidney (p=0.03) (Fig.2A). These values were not different from control.

Representative blot for COX1 and actin are shown in Fig.2B

**Protein expression for COX2.** COX2 was detected at 74 kd. The ratio COX2/actin expression in control group was 1.1 ± 0.04. The COX2/actin expression ratio was higher in both kidneys of 2K1C group. The ratio COX2/actin expression was higher than in controls in both clipped kidney (1.6 ± 0.12, p=0.006) and non-clipped kidney (1.3 ± 0.04, p=0.009) when compared to control (Fig.3A). In the TTA treated 2K1C group, the ratio COX2/actin expression was similar to controls, (1.2 ± 0.15 in the clipped kidney and 1.1 ± 0.09 in the non-clipped kidney, p≤0.02 when compared to 2K1C) (Fig.3A). Representative blot for COX2 and actin are shown in Fig.3B.

**PGE$_2$ urine and renal cortex concentration.** The PGE$_2$ level in the kidney cortex of the control group was 171 ± 10 pg g$^{-1}$. In the clipped kidney of 2K1C group, the PGE$_2$ level was 288 ± 26 pg g$^{-1}$ (p=0.001 compared to controls) and 198 ± 4 pg g$^{-1}$ in the non-clipped kidney (p=0.03 compared to controls). In the 2K1C TTA treated group, the PGE$_2$ levels were lower both in clipped (173 ± 19 pg g$^{-1}$, p<0.001 compared to 2K1C) and non-clipped kidney (170 ± 11 pg g$^{-1}$, p<0.001 compared to 2K1C), but the levels in 2K1C TTA kidneys were similar to controls in both clipped (Fig.4).

The PGE$_2$ concentration in urine from the control group was 2.4 ± 0.05 pg ml$^{-1}$. The PGE$_2$ concentration was 3.5 ± 0.1 pg ml$^{-1}$ in the urine from the 2K1C hypertensive rats (p<0.001, compared to controls). After TTA treatment, PGE$_2$ concentration was similar to
control values \((2.4 \pm 0.05 \text{ pg ml}^{-1})\) and significantly lower when compared to 2K1C \((p<0.001)\) (Fig.5).

Real-time quantitative RT-PCR for PPARs.

The mRNA levels of all PPARs were measured in both the clipped and the non-clipped kidney. The effect of TTA treatment increased the mRNA level of PPAR\(\delta\) in the non-clipped kidney. There was a tendency for a lowering effect of TTA on PPAR\(\alpha\) and PPAR\(\delta\) in clipped kidneys (Fig. 6).

Infusion of a COX2 inhibitor. Four weeks of infusion of the COX 2 inhibitor did not change blood pressure which was \(174 \pm 5 \text{ mmHg}\) after infusion compared to \(176 \pm 4 \text{ mmHg}\) in the untreated 2K1C hypertensive animals.

RBF response to ANG II after indomethacin treatment. Baseline RBF before indomethacin treatment was \(6.3 \pm 0.4 \text{ ml min}^{-1} \text{ g}^{-1}\) in the left kidney of controls, \(6.4 \pm 0.2 \text{ ml min}^{-1} \text{ g}^{-1}\) in the non-clipped kidney of the 2K1C group and \(6.5 \pm 0.2 \text{ ml min}^{-1} \text{ g}^{-1}\) in the non-clipped kidney of the TTA treated group. Inhibition of the COX system by indomethacin did not change baseline RBF or blood pressure in any of the groups.

ANG II injected into the renal artery decreased RBF in all groups. In the control group the RBF decrease was accentuated from \(21 \pm 3 \%\) before to \(44 \pm 6 \%\) after indomethacin \((p=0.002)\). In the non-clipped kidney of 2K1C, the RBF decrease was also strengthened from \(10 \pm 2 \%\) before to \(19 \pm 2 \%\) after indomethacin treatment \((p=0.003)\) (Fig. 7). TTA treatment
blunted this effect. The RBF response to ANG II was 20 ± 3 % before and 23 ± 4 % after indomethacin (p=0.1) in the TTA treated 2K1C animals (Fig.7).

DISCUSSION

The main new information in the present study is that TTA, a modified fatty acid, attenuated COX2 activity and protein expression in both clipped and non-clipped kidneys from rats with 2K1C hypertension. This may affect renal vascular reactivity and have effects on the regulation of renal hemodynamics. However, it does not appear to be the major mechanism for TTAs effect on blood pressure as neither acute general COX inhibition nor chronic specific COX2 inhibition was able to lower blood pressure.

As shown before(19, 24, 27), we found that COX2 activity was enhanced in the clipped kidney and, to some extent, also in the non-clipped kidney of untreated rats (33). In contrast, COX1 expression was unchanged in both kidneys of 2K1C, and the activity compared to total COX activity was reduced in proportion to the increase in COX2 activity. There may be a component of direct COX1 down regulation in our model, as there is a tendency towards lower values in both kidneys that corroborates results from other investigators (50). Interestingly, the TTA treatment normalized the activity of both COX1 and COX2 in both kidneys again indicating that TTA affects a common controller up-stream in the 2K1C model. The results for COX activity was borne out in that the renal content of PGE2 was nearly doubled in the clipped kidney when compared to the non-clipped kidney and PGE2 urinary output was increased in utreated 2K1C when compared to controls. Further TTA treatment normalized both the cortex level and the urinary output of PGE2 showing that the effect of TTA on COX2 expression and activity is not buffered at some other point in the system.
As previously shown the RBF response to ANG II injection into the renal artery of the non-clipped kidney was increased by indomethacin pretreatment both in controls (15) and as we have shown in isolated vessels (22), in 2K1C animals. However, in TTA treated 2K1C animals indomethacin did not alter the magnitude of the RBF response. This further supports that TTA down regulates COX2 activity and PG production, which has important effects on the renal vascular reactivity.

In the present study, we have also confirmed that TTA partially prevents the increase of blood pressure in 2K1C rats as we have shown previously (5, 16). The fact that neither indomethacin nor parecoxib has any effect on blood pressure in 2K1C rats further supports that TTAs effect on blood pressure is at an up-stream site, potentially renin expression and release. This is consistent with our previous results showing that TTA has no effect on blood pressure in spontaneously hypertensive rats that have low plasma renin concentration, as well as with earlier studies showing that renin gene expression and COX2 expression has a parallel expression in the rat cortex in renal hypertension (21). However, COX2 inhibition has been found to lower blood pressure in renovascular hypertension in 2K1C rats (33) and in rats with aortic coarction (56), which is contrary to our results, while others have found no effect of COX2 inhibition on blood pressure (41). These differences must for the time being be attributed to differences in models and strains, but may also indicate COX2 independent effects of some of the inhibitors used. This clearly warrants further study.

TTA exerts various effects via peroxisome proliferation-activated receptor (PPAR) dependent and independent pathways (3) (3, 14). The blood pressure lowering effect of other PPAR activating drugs, such as the PPARγ ligand thiazolidinedione, as reviewed previously (9, 13, 42), is linked to renin expression as we and others have shown previously (16, 44).
However, the precise mechanism is not clear as PPARγ activation in human renin producing cells in vitro has been shown to increase renin transcription (51, 52). The effect of PPAR activation on COX2 activity is consistent with previous findings in the kidney (46) and reinforces that TTA may work through a PPAR dependent pathway in affecting blood pressure in the 2K1C rat. In the present study we observed an increase of PPAR delta mRNA in the non clipped kidney, and there was a tendency towards lower expressions of both PPAR alpha and delta in the clipped kidney. No effect was seen on PPAR gamma. These results suggest that PPARs are indeed expressed in the kidney and that PPARγ, which is most closely connected to blood pressure regulation, is not significantly changed by TTA treatment.

The COX enzymes catalyse prostaglandin synthesis from free arachidonic acid (48, 53). There are three COX isoenzymes that are encoded by different genes, COX1, COX2 and COX3 (11) which transform arachidonic acid in a two-step reaction into PGG2/PGH2 further metabolized to a variety of prostanoids by specific enzymes (47, 53). COX2 is the inducible form connected with different pathological situations that activates inflammatory pathways (19) although it is well known that COX2 is also constitutionally expressed in the thick ascending limb of the loop of Henle, medullary interstitial cells and macula densa cells (19, 48). COX2 is up-regulated in both clipped and non-clipped kidney of 2K1C (24), as we found six weeks after clipping in the present study. We have previously shown that COX2 inhibition does not affect the reactivity of isolated afferent arterioles to ANGII (22). Thus it is reasonable to suggest that the effects we describe on RBF regulation is related to changes of COX2 activity in the macula densa with corresponding effects on renin regulation (38) and renal autoregulation (12, 18, 37).
An alternative explanation is that the increased PG production is related to renal hypertensive damage and inflammation which is then decreased when TTA lowers blood pressure. It has been reported that PGE2 production in inflammation is COX2 dependent (48), and in a recent study (6) we have shown that the inflammatory marker NF-kB is activated in 2K1C hypertension in both kidneys. This is of interest since it has been shown that nuclear factor kappa beta NF-kB dependent activation of IL1β and TNFα, leads to COX2 activation (54). There is also evidence regarding IL1β and TNFα dependent COX2 stimulation (39) and we have found up-regulation of TNFα and IL1β concentrations both in plasma and in kidney tissue in 2K1C rats (6).

Another mechanism that could play a role in the changes in PG concentrations could be the fact that TTA has been shown to up-regulate dihomo-γ-linolenic acid (DGLA) and down-regulate eicosapentaenoic acid (EPA) in serum of 2K1C rats, whereas the serum level of arachidonic acid (AA) was not affected. (16). DGLA, EPA and AA are competitive substrates for COX2 (49), which could have effects beyond the simple effects on expression.

**Perspectives and significance**

From the presented results we conclude that TTA down-regulates COX2 synthesis in both clipped and non-clipped kidney in a 2K1C hypertensive rat model which has potentially important effects on the regulation of RBF. The inability of indomethacin and parecoxib to reduce blood pressure indicates that TTAs effect on COX2 does not play a role in the effect on blood pressure. Based on findings that TTA prevents high blood pressure and reduces the activity of COX2, and previous findings demonstrating that TTA reduces the activity of the renin-angiotensin system. NF-kB and cytokines (7), TTA could be a promising drug for patients with renovascular hypertension.
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LEGENDS TO FIGURES

FIG.1. The systolic blood pressure before and after clipping in control, 2K1C and 2K1C TTA groups (N=6 in each group). * p<0.001 indicates the increase of blood pressure in 2K1C group compared to control. ** p<0.001 indicates the increase of blood pressure in TTA-treated group compared to control. The blood pressure in the TTA treated group was significantly lower than in untreated 2K1C group after 3 weeks of treatment.

FIG.2 The COX1 protein expression (2A, 2B) and COX1 activity (2C) in the kidney cortex in control, 2K1C and 2K1C TTA groups (N=6 in each group). In Fig. 2A *p=0.03 indicates the increased COX1 protein expression in 2K1C TTA treated group compared to 2K1C. Fig 2B shows representative Western Blot for COX1 and actin detection. In fig. 2C, * p<0.01 indicates the reduction of COX1 activity in the clipped and non-clipped kidney of 2K1C group compared to control. ** p≤0.02 indicates the enhancement of COX1 activity after TTA treatment in both clipped and non-clipped kidney compared to 2K1C. The TTA treated group is not different from the control group.

FIG.3 The COX2 protein expression (2A, 2B) and COX2 activity (2C) in the kidney cortex control, 2K1C and 2K1C TTA groups (N=6 in each group). In Fig.2A *p<0.001 indicates the up-regulation of COX2 protein expression in both clipped and non-clipped kidney compared to control, and **p≤0.02 indicates the reduction of COX2 protein expression after TTA treatment in both kidneys compared to 2K1C. Fig 3B shows representative Western Blot for COX2 and actin detection. In Fig.3C * p<0.02 indicates the reduction of COX2 activity in the clipped and non-clipped kidney of 2K1C group, both compared to control. ** p<0.04 indicates the enhancement of COX1 activity after TTA treatment in both clipped and non-
clipped kidney compared to 2K1C. The TTA treated group is not different from the control group.

**FIG.4.** The PGE$_2$ levels in the kidney cortex from control, 2K1C and 2K1C TTA groups (N=6 in each group). * p≤0.03 indicates the increased PGE$_2$ concentration in the clipped and non-clipped kidney of 2K1C group, when compared with control. **p<0.001 indicates reduction of PGE$_2$ concentration in both kidneys compared to 2K1C.

**FIG.5** The PGE$_2$ urine concentration in control, 2K1C and 2K1C TTA groups (N=6 in each group) * p<0.001 indicates the increased PGE$_2$ urine concentration in 2K1C hypertensive group compared with control. **p<0.001 indicates the reduction of PGE$_2$ urine concentration after TTA treatment compared to 2K1C.

**FIG. 6.** The mRNA levels of PPARs in clipped and non-clipped kidneys, 2K1C (open bars) and 2K1C TTA (filled bars) (N=5 in each group). In Fig.6B *p<0.05 indicates the up-regulation of PPARδ mRNA level in the non-clipped kidney after TTA treatment. The results are presented relative to 18S rRNA and normalized to clipped controls, arbitrary units.

**FIG.7** The RBF response to 2.5 ng ANG II injected in the renal artery of the non-clipped kidney in all groups. * p<0.003 indicates increased RBF response to ANG II after indomethacin treatment in control and 2K1C hypertensive group.
Fig. 1
FIG. 2

Panel A: COX1 protein expression

Panel B: COX1 and actin protein expression

Panel C: COX1 activity in the renal cortex (% from the total COX activity)
FIG. 3

COX2 protein expression

<table>
<thead>
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<th>2K1C</th>
<th>TTA</th>
<th>2K1C nc</th>
<th>TTA nc</th>
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<td>COX2</td>
<td>1.0</td>
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<td>1.4</td>
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COX2 activity in the kidney cortex (% from the total COX activity)

<table>
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<tr>
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<th>Control</th>
<th>2K1C</th>
<th>TTA</th>
<th>2K1C nc</th>
<th>TTA nc</th>
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<tbody>
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<td>10</td>
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<td>30</td>
<td>40</td>
<td>50</td>
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</tbody>
</table>

COX2 actin

* p < 0.05
** p < 0.01

the clipped kidney | the non-clipped kidney
FIG. 4

PGE2 renal cortex levels (pg.g⁻¹)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>2K1C</th>
<th>2K1C TTA</th>
<th>2K1C nc</th>
<th>2K1C TTA nc</th>
</tr>
</thead>
<tbody>
<tr>
<td>the clipped kidney</td>
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<td></td>
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<tr>
<td>the non-clipped kidney</td>
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</table>

* ** ***
FIG. 5
FIG. 6
FIG. 7

Control 2K1C TTA

RBF response to exogenous ANG II (% from the basic line)

Before Indomethacin
After Indomethacin
REFERENCES


