JPET Fast Forward. Published on May 6, 2005 as DOI: 10.1124/jpet.104.076927

Title Page

Effect of Tempol on Renal Cyclooxygenase Expression and Activity In Experimental Diabetes in the Rat

Jing Li, Yu-Jung Chen and John Quilley

Department of Pharmacology

New York Medical College

Valhalla, NY 10595

Running title page

Running title: Tempol, oxidative stress and renal COX-2 in diabetes.

Corresponding author: John Quilley, PhD

Department of Pharmacology

New York Medical College

Valhalla, NY 10595

Tel. 914 594-4123

FAX 914 347-4956

E-mail john_quilley@nymc.edu

Text pages: 16

Figures: 7

References: 29

Words in: Abstract 240

Introduction 502

Discussion 1534

Abbreviations: AA – arachidonic acid; COX – cyclooxygenase; STZ – streptozotocin; SOD – superoxide dismutase

Section: Cardiovascular

JPET Fast Forward. Published on May 6, 2005 as DOI: 10.1124/jpet.104.076927
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Renal COX-2 expression is increased in the streptozotocin (STZ)-diabetic rat and is associated with enhanced renal prostaglandin release in response to arachidonic acid (AA). Endoperoxide-mediated vasoconstrictor responses to AA were also enhanced in the diabetic rat kidney. As oxidative stress is increased in diabetes and has been shown to induce COX-2, we assessed its contribution to prostaglandin release by treating diabetic rats with tempol (120mg/kg/day) for 28 days. Release of AA-stimulated prostaglandins, PGE_2 and 6-ketoPGF$_{1\alpha,}$ from the isolated perfused kidney was used as an index of COX activity and Western analysis was used to determine COX-2 protein expression. In untreated diabetic rats, the release of prostaglandins in response to AA was markedly enhanced; the increase in release of both 6-ketoPGF$_{1\alpha}$ and PGE_2 after AA was twice that seen in control rats. Renal cortical COX-2 expression in diabetic rats was 3-fold that of control rats. Tempol treatment reduced the AA-stimulated release of prostaglandins to levels seen in control rats; this was associated with reduced expression of COX-2 protein to levels not different from control rats. However, the enhanced vasoconstrictor response to AA in diabetic rats was unaffected by tempol treatment but abolished by inhibition of COX-1 with SC58560. The addition of tempol to the perfusate of kidneys from diabetic and control rats had only a slight effect on prostaglandin release. We conclude that oxidative stress is an integral component of the mechanism involved in the induction of renal COX-2 in diabetes.

Introduction

Studies in both human and experimental diabetes mellitus have revealed alterations of renal arachidonic acid (AA) metabolism and cyclooxygenase (COX)-dependent prostaglandin and thromboxane production. For the most part, these studies showed an increase in COX activity and prostaglandin release from the kidney, mesangial cells and glomeruli of diabetic rats (Kreisberg and Patel, 1983; Schambelan et al, 1985; Quilley and McGiff, 1985). Craven et al (1987) linked these changes to the increased GFR observed in diabetes by showing that inhibition of COX with indomethacin reduced GFR in diabetic but not control rats. However, most of these studies were conducted before the recognition and characterization of multiple COX isoforms. Recent studies indicate that COX-2 may contribute to the increased formation of renal prostaglandins in the diabetic rat and that these may play a role in the renal functional consequences. Thus, Komers et al. (2001) reported increased renal cortical expression of COX-2 and found that administration of a COX-2 inhibitor reduced GFR in streptozotocin (STZ)-diabetic rats treated with insulin. Harris' group reported that chronic administration of a COX-2 inhibitor to hypertensive diabetic rats reduced the appearance of markers for renal damage, suggesting that increased COX-2 may be an early step in the development of nephropathy (Cheng et al. 2002). We confirmed the increase in the renal cortical expression of COX-2 in untreated STZ-diabetic rats and showed that a COX-2 inhibitor reduced the enhanced endoperoxide-mediated renal vasoconstrictor response to AA as well as the enhanced associated release of prostaglandins that we had reported for the diabetic rat (Quilley and McGiff, 1990; Quilley and Chen, 2003).

The stimulus for induction of COX-2 in diabetes is likely hyperglycemia, which has been shown to increase oxidative stress in a variety of cells including endothelial cells and mesangial cells (Catherwood et al. 2002; Cosentino et al. 2003). Moreover, diabetes is well recognized as a condition of oxidative stress and reactive oxygen species such as superoxide have been shown to induce COX-2 (Kiritoshi et al. 2003; Cosentino et al. 2003). However, the role of oxidative stress and superoxide in the induction of renal COX-2 in diabetes has not been addressed, the primary aim of the studies reported here. Consequently, we addressed the effects of chronic administration of the superoxide dismutase (SOD) mimetic, tempol, on the induction of renal COX-2 in the STZ-diabetic rat. Renal release of prostaglandins in response to AA was used as an index of COX activity although it does not distinguish the activity of COX-2 from COX-1. The results confirmed those of our earlier study, showing increased renal expression of COX-2, which was associated with increased release of prostaglandins in response to AA in the diabetic rat. In addition, the renal vasoconstrictor effect of AA, which is COX-dependent and mediated via endoperoxides (Quilley et al., 1989) was enhanced in the diabetic rat. Treatment with tempol to reduce superoxide prevented the increased renal expression of COX-2 and reduced AA-stimulated release of prostaglandins in the diabetic rat but did not influence the enhanced vasoconstrictor effect of AA.

Methods

These studies were conducted in accordance with NIH guidelines for the care and use of laboratory animals. Diabetes was induced in 7 week old male Wistar rats, weight 200-220g, with STZ (70mg/kg i.v.) in citrate buffer, pH 4.5. Age- and weight-matched control rats were given an equivalent volume of the vehicle, citrate buffer. 48 hours after STZ treatment, half of the diabetic rats were treated the SOD mimetic, tempol, which was added to the drinking water (1mmol/L). As the water intake of diabetic rats is markedly increased, they received approx. 120mg/kg/day tempol which is higher than that employed in most other studies, eg, Sedeek et al. (2003) employed tempol at approx. 30mg/kg/day and showed that it reduced urinary excretion of isoprostanes in both control and endothelin-treated rats. The dose that we used is comparable to that used by Nassar et al. (2002) who reported that tempol treatment abolished the increase in vascular superoxide and isoprostane formation in diabetic rats. The second group of diabetic rats and the control group received no treatment.

28 days later, rats were anesthetized with pentobarbitone (65mg/kg ip.) and the kidney prepared for perfusion. Briefly, following a midline laparotomy, the right renal artery was cannulated via the mesenteric artery to avoid interruption of blood flow and the kidney removed from the rat and perfused with oxygenated Krebs' Henseleit buffer at 37^{o} C at constant flow, which was adjusted to obtain a perfusion pressure of 60-90mmHg. Glucose levels in tail vein blood were determined with a glucometer (Ames). The left kidney was removed for determination of renal cortical COX-2 protein expression.

After at least 10 min. perfusion and once a stable perfusion pressure was obtained, vasoconstrictor responses to 0.3, 1 and 3μg AA were determined. Responses to a single

dose of angiotensin II (10ng) were also determined. Perfusate samples were collected for 1min. before and 1min. after the 3μg dose of AA to determine prostaglandin release.

In another series of experiments to address the acute effects of tempol on renal AA responses and prostaglandin release, we used diabetic and control rats 8-10 weeks after the induction of diabetes with STZ or treatment with vehicle, respectively. The kidneys were isolated and perfused with Krebs' buffer containing tempol (500μM) or vehicle (water) and perfusion pressure responses to 1, 3 and 10μg AA were determined. Samples for prostaglandin release were obtained as described above. The doses of AA chosen were higher than those in the earlier experiments as these rats were slightly older (15-17weeks) and sensitivity to the vasoconstrictor effects of AA declines with age. (Quilley and McGiff, 1990). However, regardless of age, the diabetic rat kidney exhibits an exaggerated response to AA compared to age-matched control rats.

In another series of experiments, we tested the effects of a selective inhibitor of COX-1 on the renal vasoconstrictor effects of AA and the associated release of prostaglandins. Rats with diabetes of 4-6 weeks duration and age-matched control rats were used. The isolated kidney was prepared as described above and the COX-1 inhibitor, SC58560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole), which was a gift from Pfizer, was added to the perfusate at a concentration of 5×10^{-8}M, which is almost 20 times the IC50 for recombinant COX-1 and almost 100 times less than the IC50 for recombinant COX-2 (Smith et al., 1998). At least 15min was allowed before responses to 1 and 3μg AA were determined. Responses to phenylephrine (100ng) were also tested to determine any effects of the inhibitor in reducing vasoconstrictor responsiveness.

JPET Fast Forward. Published on May 6, 2005 as DOI: 10.1124/jpet.104.076927
This article has not been copyedited and formatted. The final version may differ from this version.

Prostaglandin measurements. One minute perfusate collections were made immediately before and after the administration of 3μg AA for the measurement of PGE_2 and 6-keto$PGF_{1\alpha}$ as an index of cyclooxygenase activity. Levels of the two prostanoids in the renal perfusates were determined by enzyme immunoassay using kits obtained from Cayman Chemical Company. 6-Keto$PGF_{1\alpha}$ was chosen as an index of conversion of AA by the endothelium, the presumed site of generation of endoperoxides, whereas PGE_2 levels were determined as an index of total renal prostaglandin formation.

Urinary isoprostane excretion. Another group of rats with diabetes of 5 weeks duration were compared to age-matched control rats for urinary isoprostane excretion. Rats were placed in metabolism cages and 24hour urines were collected. 8-isoprostane $PGF_{2\alpha}$ was measured using an enzyme immunoassay (Cayman). Unpurified samples were diluted in EIA buffer for assay.

Western blot. The cortex was homogenized in RIPA buffer and subjected to centrifugation at 10,000 and 14,000 rpm. The protein in the supernate was determined using a Bio-Rad assay kit and 50μg was mixed with 5X SDS-PAGE sample buffer (500mmol/L DTT. 0.2% bromophenol blue and 50% glycerol) and boiled for 3 mins. Proteins were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with a rabbit anti-mouse COX-2 polyclonal antibody (1:1000 dilution; Caymen chemical company). Membranes were washed with Tris-buffered saline containing Tween 20 and incubated with horseradish peroxidase-conjugated antisera. COX-2 protein was then detected by enhanced chemiluminescence.

Analysis of data. All data are expressed as means±SEM and were compared using an unpaired t-test or ANOVA in which individual points were compared using a modified t-statistic (Bonferroni). A value of $P<0.05$ was considered statistically significant.

Results.

When tempol treatment was initiated 48h after administration of STZ, the mean blood glucose level in this group of rats (n=5) was 501±30mg/dL. Treatment with tempol for 28 days did not affect blood glucose levels in diabetic rats, 553±25mg/dL compared to 489±33mg/dL in the untreated diabetic group (n=5), both of which were significantly greater than that of the citrate-treated control group (n=5), 126±10mg/dL ($p<0.01$). Body weights in the treated and untreated diabetic groups were 291±6 and 295±14g, respectively, which were significantly lower than that of the control group, 387±14g ($p<0.05$) when weighed after 28 days of treatment at the time of sacrifice.

For the isolated perfused kidney preparations, there were no differences in basal perfusion pressure among the various groups: 73±4, 68±2 and 67±2mmHg in citrate, diabetic and diabetic plus tempol groups respectively. However, perfusate flow rates were higher in the two diabetic groups, 15±1.4ml/min. for the untreated group and 16±1.4ml/min. for the tempol-treated group, compared to 10.8±0.4ml/min. for the control group.

The release of PGE_2 and 6-keto$PGF_{1\alpha}$ from control, diabetic and tempol-treated diabetic rat kidneys before and after 3μg AA is shown in figures 1 and 2. Basal release of PGE_2 at the beginning of the perfusion was less in the diabetic groups than the control group. In the untreated diabetic group, PGE_2 release was 883±99pg/min. compared to 611±77pg/min. for the tempol-treated diabetic group and 1481±412pg/min. for the control group. These differences became more apparent with time of perfusion and following administration of AA. There were no differences between the groups in terms of basal release of 6-keto$PGF_{1\alpha}$ at the beginning of the perfusion. However, release prior

to the administration of 3μg AA was significantly less in the tempol-treated diabetic group compared to the untreated diabetic and control groups (fig. 2). Following administration of AA, release of both PGE_2 and 6-keto$PGF_{1\alpha}$ were greater in the untreated diabetic group compared to the control group (figs. 1 and 2). In contrast, tempol treatment of diabetic rats for 28 days reduced the enhanced release of PGE_2 and 6-keto$PGF_{1\alpha}$ observed in the untreated diabetic group so there were no differences from the control group.

In these groups of rats, we also determined COX-2 protein expression in renal cortical homogenates obtained at the time of sacrifice after 28 days of treatment with tempol or no treatment. Figure 3 shows the results of Western analysis for the expression of COX-2, standardized against the expression of β-actin, in control, diabetic and tempol-treated diabetic rat kidneys. As we reported earlier, diabetes leads to increased renal COX-2 expression, which corresponds to increased release of prostaglandins stimulated by AA. Tempol treatment of diabetic rats for 28 days prevented the increase in COX-2 expression in the diabetic rat kidney so that levels were similar to those seen in the control group, also consistent with the effects of tempol on AA-stimulated prostaglandin release.

Administration of bolus doses of AA to the perfused kidney resulted in dose-dependent vasoconstrictor responses that were markedly enhanced in kidneys from diabetic rats (fig. 4). Treatment of diabetic rats with tempol for 28 days did not modify the enhanced vasoconstrictor effects of AA (fig. 4). We also determined the response to a bolus dose of 10ng angiotensin II in four of the five kidneys from each group of rats. The response was much greater in the untreated diabetic group compared to the control group,

138±12mmHg versus 36±12mmHg ($p<0.01$). Tempol treatment of diabetic rats moderated the enhanced vasoconstrictor effect of angiotensin II to 81±13mmHg.

Because tempol treatment of diabetic rats prevented the increased expression of COX-2 and the enhanced release of AA-stimulated prostaglandins, we conducted another series of experiments to determine the acute effects of tempol on AA-stimulated prostaglandin release from the isolated kidney to determine whether tempol affected COX activity. Thus, tempol was added to the perfusing solution at a concentration of 500μM. As before, perfusate flow rates were slightly higher in the diabetic rat kidneys compared to control rat kidneys; 15.6±0.6ml/min. for the untreated diabetic group, 14.9±0.5ml/min.for the tempol-treated diabetic group, 13.0±0.7ml/min. for the untreated control group and 12.9±0.4 ml/min. for the tempol-treated citrate group which produced perfusion pressures of 64±5, 63±2, 66±3 and 71±2mmHg, respectively. Figure 5 shows the release of 6-ketoPGF$_{1\alpha}$ before and after 3μg AA in the various groups. Confirming the earlier studies, basal release was not different between the diabetic and control group and was unaffected by tempol. However, AA-stimulated release of 6-ketoPGF$_{1\alpha}$ was greatly enhanced in the diabetic rat kidney compared to the control. Tempol was without influence on AA-stimulated release in the control group but resulted in a slight reduction in the diabetic group. The release of PGE_2 followed a similar trend to that of the earlier studies, i.e., basal release was reduced in the diabetic whereas AA-stimulated release was enhanced. Neither basal nor stimulated PGE_2 release was affected by tempol (data not shown). As before, the renal vasoconstrictor effect of AA was greatly enhanced in the diabetic versus the control rat. For example, 3μg AA increased perfusion pressure by 191±30mmHg in the diabetic rat kidney (n=3) compared to 37±12mmHg in the control

(n=4). Acute administration of tempol to the kidneys did not modify the vasoconstrictor effect of AA in either the diabetic (n=4) or control group (n=4), 207±19mmHg and 30±6mmHg, respectively.

As tempol treatment prevented the induction of renal COX-2 in the diabetic rat but failed to influence the vasoconstrictor effect of AA, suggesting a lack of involvement of COX-2-derived prostanoids, we conducted additional experiments with a selective inhibitor of COX-1. As before, vasoconstrictor responses to AA were enhanced in untreated kidneys of diabetic rats compared to control rat kidneys (fig 6) whereas vasoconstrictor responses to phenylephrine were not different. However, inhibition of COX-1 with SC58560 virtually abolished the vasoconstrictor effects of AA in both diabetic and control rat kidneys without affecting the response to phenylephrine. Thus, 100ng phenylephrine increased perfusion pressure by 81±20 and 95±16mmHg in control and diabetic rat kidneys, respectively (n=4-5). In the presence of SC58560, the increases in perfusion pressure were 73±20 and 61±17mmHg in control and diabetic rat kidneys, respectively. Fig 7 shows the effects of SC58560 on PGE_2 release before and after 3µg AA in control and diabetic rats. Basal release was reduced in the diabetic compared to the control rat kidney and SC58560 produced a marked decrease in basal PGE_2 release in control rat kidneys and abolished the increase in response to AA in both control and diabetic rat kidneys.

In additional experiments, we compared urinary isoprostane excretion as a marker for oxidative stress in a group of diabetic rats (n=5) and a group of age-matched control rats (n=5). Urinary isoprostane excretion was 53.7±7.8ng/day in the diabetic group compared to 26.8±4.2ng/day for the control group.

Discussion.

The results of this study clearly show that tempol treatment, initiated 48 hours after the induction of diabetes and maintained for 28 days, prevents the diabetes-induced increase in the renal expression of COX-2, which is associated with a reduction in the enhanced AA-stimulated prostaglandin release from the isolated kidney of the diabetic rat. However, the enhanced renal vasoconstrictor response to AA in the diabetic rat kidney was not affected by tempol treatment. These effects of tempol could not be attributed to improvement of the diabetic condition as blood glucose levels were unaffected.

STZ-induced diabetes increases the renal expression of COX-2 protein (Komers et al. 2001; Quilley and Chen, 2003). We showed that the increase in COX-2 expression was associated with increased release of prostaglandins from the kidney challenged with AA as well as enhanced vasoconstrictor responses to AA (Quilley and Chen, 2003). We suggested that COX-2 contributed to both of these manifestations of experimental diabetes as an inhibitor of COX-2 reduced the vasoconstrictor effects of the lower doses of AA only in the diabetic rat kidney and also reduced the release of prostaglandins. The present study provides evidence for oxidative stress associated with diabetes as the stimulus for induction of COX-2 as tempol completely prevented the increase in COX-2 expression in diabetic rats, which exhibit increased oxidative stress using urinary isoprostane excretion as a marker. This is consistent with in vitro studies demonstrating that oxidative stress increases COX-2 protein expression in a variety of cells (Kiritoshi et al. 2003; Cosentino et al. 2003) and that exposure of cells to elevated glucose levels increased superoxide formation and oxidative stress (Catherwood et al. 2002; Cosentino

et al. 2003). However, this is the first study that links hyperglycemia, oxidative stress and induction of COX-2 in vivo. We assume that hyperglycemia associated with the diabetic state leads to oxidative stress that, in turn, results in the induction of COX-2. Nonetheless, we cannot exclude some other manifestation of the diabetic state as a contributory factor to the induction of COX-2. As COX-2 has been linked to renal damage in models of diabetes by showing that inhibition of COX-2 reduced the expression of markers of renal injury in a hypertensive diabetic rat model and that an EP receptor antagonist also reduced renal injury (Cheng et al. 2002; Makino et al. 2002), prevention of induction of COX-2 with tempol could be expected to ameliorate the renal complications of diabetes. It would be of great interest to maintain diabetic rats on tempol and determine the progress of renal injury. If hyperglycemia is the initial stimulus for the induction of COX-2, then the effects should not be limited to the kidney but to all other cells exposed to the diabetic milieu, particularly endothelial cells of blood vessels.

As AA elicits COX-dependent, endoperoxide-mediated vasoconstrictor responses in the rat kidney that are associated with release of prostaglandins, we sought to use these an indices of COX activity. In the diabetic rat kidney, both the enhanced vasoconstrictor effect of AA and the increased release of prostaglandins corresponded with increased expression of COX-2. However, treatment with tempol, which prevented the increased expression of COX-2 revealed dissociation between the vasoconstrictor response to AA and the release of prostaglandins. Tempol treatment reduced AA-stimulated prostaglandin release without modifying the vasoconstrictor effect of AA. A similar dissociation was seen in our earlier study where inhibition of COX-2 with nimesulide in control rat kidneys reduced AA-stimulated release of prostaglandins but did not modify

the vasoconstrictor effect of AA in contrast to what was observed in diabetic rat kidneys (Quilley and Chen, 2003). However, we cannot entirely exclude an effect of nimesulide on COX-1. Because of this discrepancy we conducted experiments with a selective inhibitor of COX-1, which almost abolished the renal vasoconstrictor effect of AA in both diabetic and control rat kidneys. This effect could not be attributed to a generalized decrease in vasoconstrictor responsiveness as responses to phenyleprine were unaffected. These results indicate that COX-1 is the isoform responsible for the generation of prostanoids that elicit renal vasoconstriction in response to AA although we cannot exclude an effect of the inhibitor, which abolished AA-stimulated prostaglandin release, on COX-2. These observations are difficult to explain in light of our previous studies showing that inhibitors of COX-2 reduced prostaglandin release and vasoconstrictor responses to AA in the diabetic rat kidney but failed to modify the vasoconstrictor effect of AA in control rat kidneys. If nimesulide exerted an inhibitory effect on COX-1, vasoconstrictor responses to AA should also be reduced in control rat kidneys, which was not the case. We have no explanation for this discrepancy. Based on the present study, COX-1 ***is*** the isoform responsible for the conversion of AA to the endoperoxides in the renal vasculature of both non-diabetic rats and diabetic rats and an increase in expression or activity could account for the increased vasoconstrictor responses to AA. COX-2 is expressed constitutively at several sites in the kidney (Harris et al., 1994; Davidge, 2002) where it presumably contributes to the formation of prostaglandins in response to AA given intravascularly. However, in the diabetic kidney, it is possible that infiltration of inflammatory cells contribute to the increase in COX-2. We cannot assume that prostaglandin release from the perfused kidney is derived solely from the vasculature,

which is the site of action for the vasoconstrictor effect of endoperoxides, formed as the result of activity of COX-1. It would seem likely that the vasculature is one of several sources of prostaglandins produced by the kidney in response to AA and, therefore, release in response to AA may not always be expected to correlate with vasoconstrictor activity. Moreover, differences in vasoconstrictor responsiveness to endoperoxides in diabetic versus control rat kidneys could also explain increased vasoconstrictor responsiveness to AA in the absence of increased production in the diabetic rat. We have shown that renal vasoconstrictor responses to U46619, an endoperoxide analogue, are enhanced in the diabetic rat (Quilley and McGiff, 1990). Consequently, vasoconstrictor responses to AA do not necessarily provide a good index of COX activity. However, release of prostaglandins in response to AA is a useful index of renal COX activity but does not provide information on the site of increased expression/activity of COX. Immunohistochemical analysis of COX-1 and COX-2 is required to determine the sites of increased COX-2 expression.

The observation that tempol treatment reduced AA-stimulated prostaglandin release in conjunction with reduced renal expression of COX-2 suggests that COX-2 contributes to the formation of prostaglandins in the diabetic rat kidney. However, it is also possible that tempol, by reducing oxygen-derived free radicals, may have a direct effect on COX activity which requires the presence of hydroperoxides for catalytic activity (Davidge, 2001). We addressed this possibility by testing the acute effects of tempol**,** added to the renal perfusate, on responses to AA and measuring the release of prostaglandins. In samples obtained immediately before the administration of AA, tempol was without effect on prostaglandin release from either control or diabetic rat kidneys.

This is in contrast to chronic administration of tempol to diabetic rats where basal renal release of prostaglandins was decreased compared with untreated diabetic rats. However, treatment of diabetic rat kidneys with tempol had a slight inhibitory effect on AA-stimulated prostaglandin, achieving significance with 6-keto$PGF_{1\alpha}$. These results indicate that tempol may influence the activity of COX when it is maximally stimulated and could account, in part, for the diminished AA-stimulated prostaglandin release in kidneys of diabetic rats treated with tempol for 28 days, assuming that tempol remains within the cells after perfusion of the kidney is started.

The mechanism whereby diabetes-induced oxidative stress increases the renal expression of COX-2 was not addressed in this study and awaits future investigation. Protein kinase C has been invoked but it is not clear whether hyperglycemia-induced activation of this kinase leads to oxidative stress or whether oxidative stress increases the activity of protein kinase C (Nishikawa et al., 2000; Whiteside and Dlugosz, 2002). Similarly, induction of COX-2 in response to cytokines and LPS, which stimulates the production of cytokines, involves oxidative stress (Feng et al., 1995). As a feature of diabetes is altered cytokine formation (Kalantarinia et al., 2003), the sequence of events remains to be clarified. The results of this study do not mean that superoxide is the mediator of COX-2-induction, only that is plays a central role. Thus, NO formation is also increased in the diabetic kidney (Sugimoto et al., 1998; Onozato et al., 2002; Cosenzi et al., 2002)) and its product upon reaction with superoxide, i.e., peroxynitrite, has also been reported to induce COX-2 (Eligini et al., 2001; Nedelec et al., 2001). That peroxynitrite formation is increased in diabetes is apparent from increased staining for nitrotyrosine (Thuraisingham et al., 2000; Onozato et al., 2002) and preliminary data

from our laboratory showing increased renal expression of nitrotyrosine support these results.

In summary, this study shows that the induction of renal COX-2 in diabetes can be prevented by tempol treatment, which coincidentally reduces AA-stimulated prostaglandin release from the kidney without modifying the vasoconstrictor effect of AA, which appears to be solely dependent on COX-1 activity. The results with tempol, therefore, suggest a role of superoxide, which is increased by diabetes and hyperglycemia, in the induction of COX-2.

References

Cheng HF, Wang CJ, Moeckel GW, Zhang MZ, McKanna JA and Harris RC (2002) Cyclooxygenase-2 inhibitor blocks expression of mediators of renal injury in a model of diabetes and hypertension. Kid Int **62**: 929-939.

Cosentino F, Eto M, De Paolis P, van der Loo B, Bachschmid M, Ullrich V, Kouroedov A, Gatti CD, Joch H, Volpe M, Luscher TF (2003) High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells. Role of protein kinase C and reactive oxygen species. Circulation **107**: 1017-1023.

Cosenzi A, Bernobich E, Bonavita M, Trevisan R, Bellini G, and Campanacci L (2002) Early effects of diabetes on inducible nitric oxide synthase in the kidney. Acta Diabetol **39**: 91-96.

Catherwood MA, Powell LA, Anderson P, McMaster D, Sharpe PC, and Trimble ER (2002) Glucose-induced oxidative stress in mesangial cells. Kidney Int **61**: 599-608.

Craven PA, Caines MA, and DeRubertis FR (1987) Sequential alterations in glomerular prostaglandin and thromboxane synthesis in diabetic rats: relationship to the hyperfiltration of early diabetes. Metabolism **36**: 95-103.

Davidge ST (2001) Prostaglandin H synthase and vascular function. Circ Res **89**: 650-60.

Eligini S, Habib A, Lebret M, Creminon C, Levy-Toledano S, and Maclouf J (2001) Induction of cyclo-oxygenase-2 in human endothelial cells by SIN-1 in the absence of prostaglandin production. Br J Pharmacol **133**: 1163-1171.

Feng L, Xia Y, Garcia GE, Hwang D, and Wilson CB (1995) Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. J Clin Invest **95**: 1669-1675.

Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, and Breyer MD (1994) Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. J Clin Invest **94**: 2504-2510.

Kalantarinia K, Awad AS and Siragy HM (2003) Urinary and renal interstitial concentrations of TNFα increase prior to the rise in albuminuria in diabetic rats. Kid Int **64**: 1208-1213.

Kiritoshi S, Nishikawa T, Sonoda K, Kukidome D, Senokuchi T, Matsuo T, Matsumura T, Tokunaga H, Brownlee M and Araki E (2003) Reactive oxygen species from mitochondria induce cyclooxygenase-2 gene expression

in human mesangial cells: potential role in diabetic nephropathy. Diabetes **52**: 2570-2577.

Komers R, Lindsley JN, Oyama TT, Schutzer WE, Reed JF, Mader SL, and Anderson S (2001) Immunohistochemical and functional correlations of renal cyclooxygenase- 2 in experimental diabetes. J Clin Invest **107**: 889-898.

Kreisberg JI and Patel PY (1983) The effects of insulin, glucose and diabetes on prostaglandin production by rat kidney glomeruli and cultured gloerular mesangial cells. Prostaglandins Leukotriene Med **11**: 431-442.

Martinez J & Moreno JJ (2000) Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem Pharmacol **59**: 865-870.

Makino H, Tanaka I, Mukoyama M, Sugawara A, Mori K, Muro S, Suganami T, Yahata K, Ishibashi R, Ohuchida S, Maruyama T, Narumiya S, Nakao K (2002) Prevention of diabetic nephropathy in rats by prostaglandin E receptor EP_1-selective antagonist. J Am Soc Nephrol **13**: 1757-1765.

Nassar T, Kadery B, Lotan C, Da'as N, Kleinman Y, and Yehia A (2002) Effects of the superoxide dismutase-mimetic compound tempol on

endothelial dysfunction in streptozotocin-induced diabetic rats. Eur J Pharmacol **436**: 111-118.

Nedelec E, Abid A, Cipolletta C, Presle N, Terlain B, Netter P, and Jouzeau J (2001) Stimulation of cyclooxygenase-2-activity by nitric oxide-derived species in rat chondrocyte: lack of contribution to loss of cartilage anabolism. Biochem Pharmacol **61**: 965-978.

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, and Brownlee M (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature **404**: 787-790.

Onozato ML, Tojo A, Goto A, Fujita T, and Wilcox CS (2002) Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. Kidney Int **61**: 186-194.

Quilley J and McGiff JC (1985) Arachidonic acid metabolism and urinary excretion of prostaglandins and thromboxane in rats with experimental diabetes mellitus. J Pharm Exp Ther **234**: 211-216.

Quilley J, McGiff JC and Najsletti A (1989) Role of endoperoxides in arachidonic acid-induced vasoconstriction in the isolated perfused kidney of the rat. Br J Pharmacol **96**: 111-116.

Quilley J & McGiff JC (1990) Renal vascular responsiveness to arachidonic acid in experimental diabetes. Br J Pharmacol **100**: 336-340.

Quilley J and Chen Y-J (2003) Role of COX-2 in the enhanced vasoconstrictor effect of arachidonic acid in the diabetic rat kidney. Hypertension **42**: 837-843.

Schambelan M, Blake S, Sraer J, Bens M, Nivez MP, and Wahbe F (1985) Increased prostaglandin production by glomeruli isolated from rats with streptozotocin-induced diabetes mellitus. J Clin Invest **75**: 404-412.

Sedeek MH, Llinas MT, Drummond H, Fortepiani L, Abram SR, Alexander BT, Reckelhoff JF, and Granger JP (2003) Role of reactive oxygen species in endothelin-induced hypertension. Hypertension **42**: 806-810.

Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JJ, Masferrer JL, Seibert K and Isakson PC (1998) Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc Natl Acad Sci USA **95**: 13313-13318.

Sugimoto H, Shikata K, Matsuda M, Kushiro M, Hayashi Y, Hiragushi K, Wada J, and Makino H (1998) Increased expression of endothelial cell nitric oxide synthase (ecNOS) in afferent and glomerular endothelial cells is involved in glomerular hyperfiltration of diabetic nephropathy. Diabetologia **41**: 1426-1434.

Thuraisingham RC, Nott CA, Dodd SM, and Yaqoob MM (2000) Increased nitrotyrosine staining in kidneys from patients with diabetic nephropathy. Kidney Int **57**: 1968-1972.

Whiteside CI and Dlugosz JA (2002) Mesangial cell protein kinase C isozyme activation in the diabetic milieu. Am J Physiol **282**: F975-F980.

Footnotes

This work was supported by a grant from the American Diabetes Association and from NIH (HL-069061).

Figure legends

Figure 1. Effect of tempol treatment for 28 days on the release of PGE_2 before and after arachidonic acid (3μg) from the isolated perfused kidney from diabetic rats; comparison with age-matched untreated diabetic (STZ) and control (citrate) rats. (n=5 for each group). * $p<0.05$ versus citrate group; # $p<0.05$ versus STZ group.

Figure 2. Effect of tempol treatment for 28 days on the release of 6-keto $PGF_{1\alpha}$ before and after arachidonic acid (3μg) from the isolated perfused kidney of diabetic rats; comparison with age-matched diabetic (STZ) and control (citrate) rats. (n=5 for each group). * $p<0.05$ versus citrate group; # $p<0.05$ versus STZ group.

Figure 3. Results of Western analysis of COX-2 protein expression, standardized against β-actin, in renal cortical samples from untreated and tempol-treated (28 days) diabetic rats and untreated control rats (n=3 for the citrate control and untreated diabetic groups and n=4 for the tempol-treated diabetic group).

Figure 4. Effect of tempol treatment of diabetic rats for 28 days on vasoconstrictor responses to arachidonic acid in the isolated perfused kidney; comparison with age-matched untreated diabetic and control rat kidneys. (n=5 for each group). * $p<0.05$ versus citrate control.

Figure 5. Effects of the addition of tempol (500μM) to the perfusate of kidneys from diabetic and age-matched control rats on the release of 6-keto$PGF_{1\alpha}$ before and after the administration of arachidonic acid (3μg). (n=3 for the untreated diabetic group and n=4 for the other groups). * $p<0.05$ versus citrate group; # $p<0.05$ versus the STZ group.

Figure 6. Effects of SC58560 (5×10^{-8}M), added to the perfusate of kidneys of diabetic (n=5) and control (n=4) rats, on vasoconstrictor responses to arachidonic acid; comparison with untreated kidneys from each group. *$p<0.05$.

Figure 7. Effects of SC58560 (5×10^{-8}M), added to the perfusate of kidneys of diabetic (n=5) and control (n=4) rats, on release of PGE_2 before and after challenge with 3μg arachidonic acid.

Fig. 1

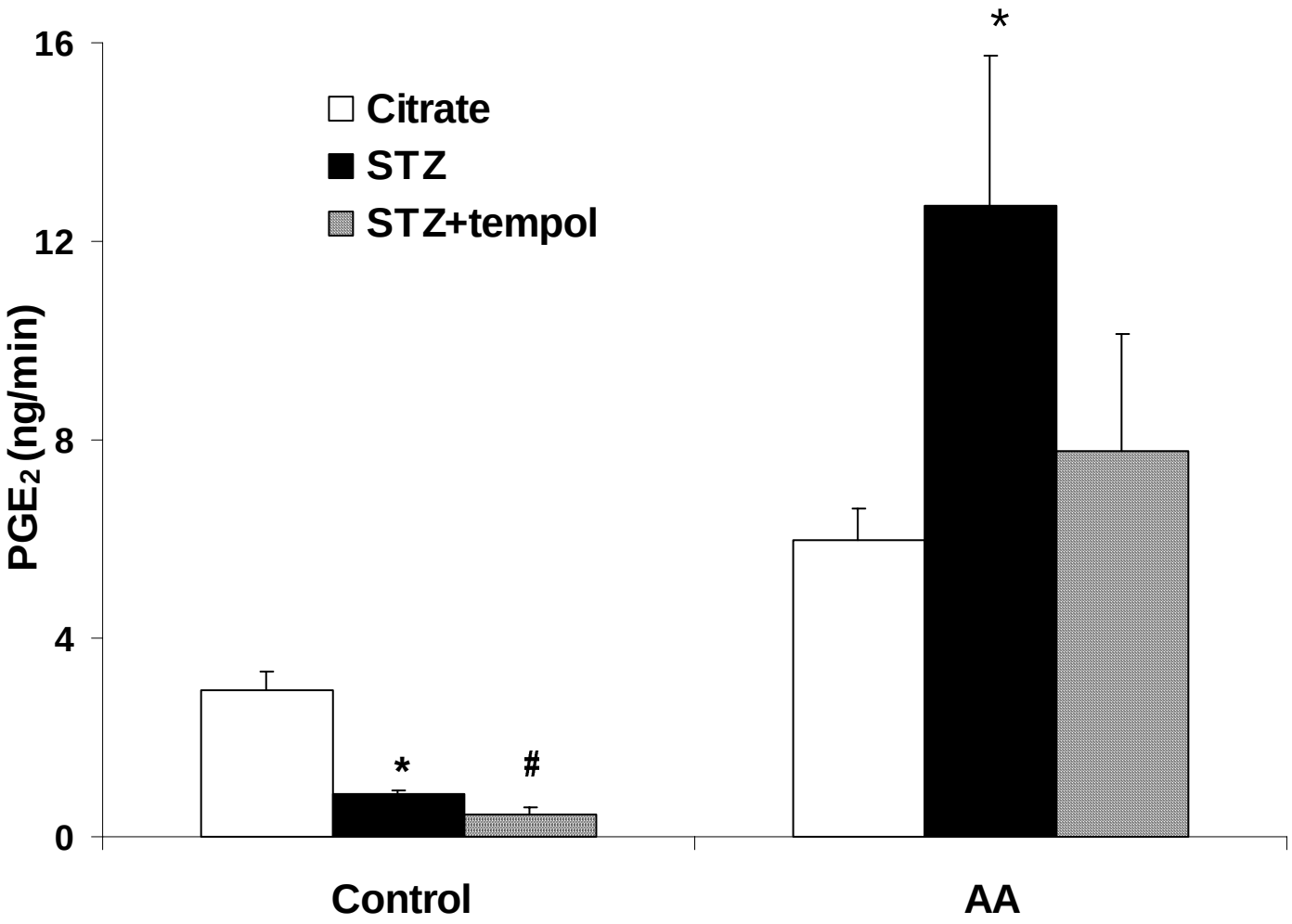

Fig. 2

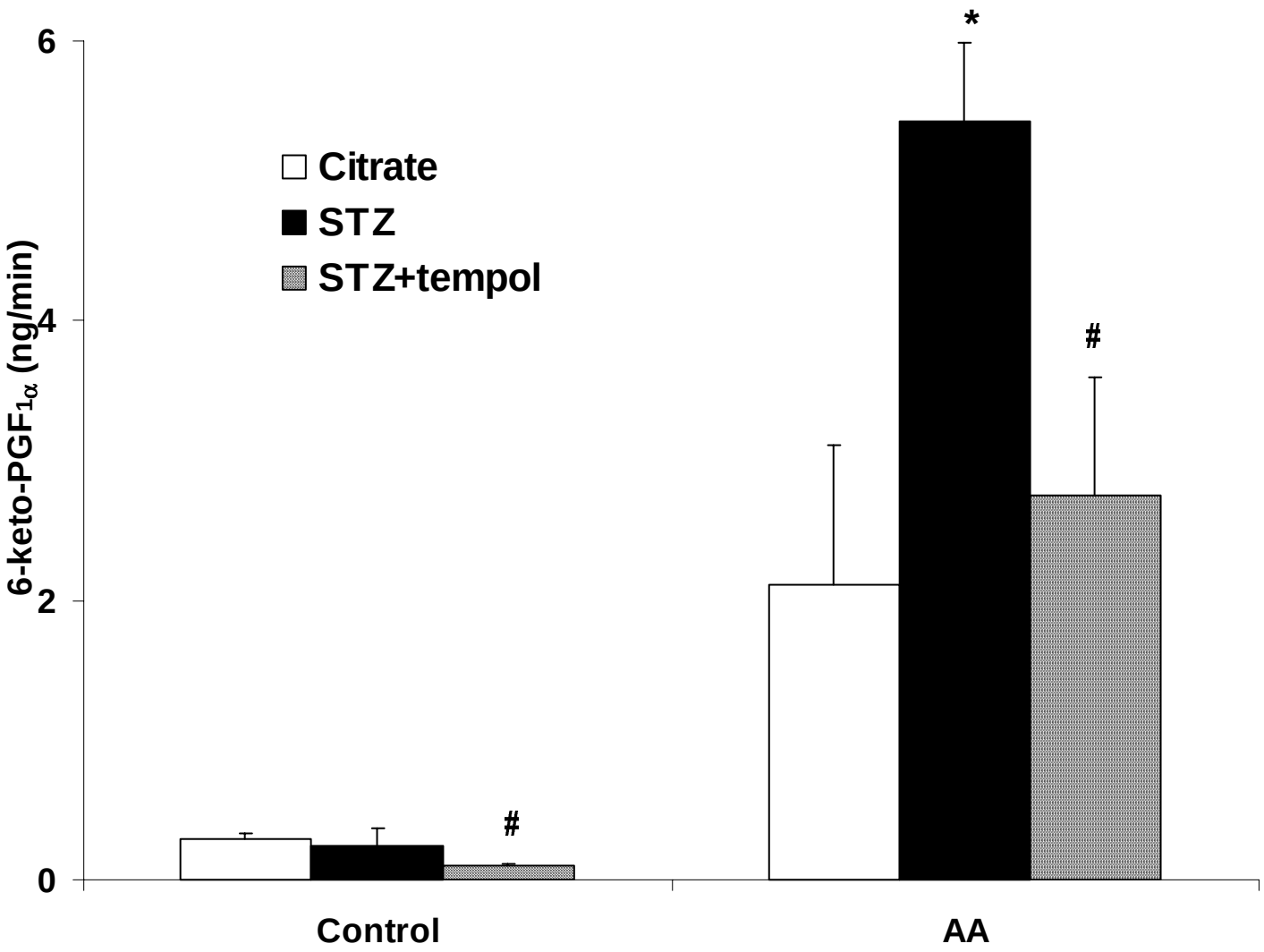

Fig. 3

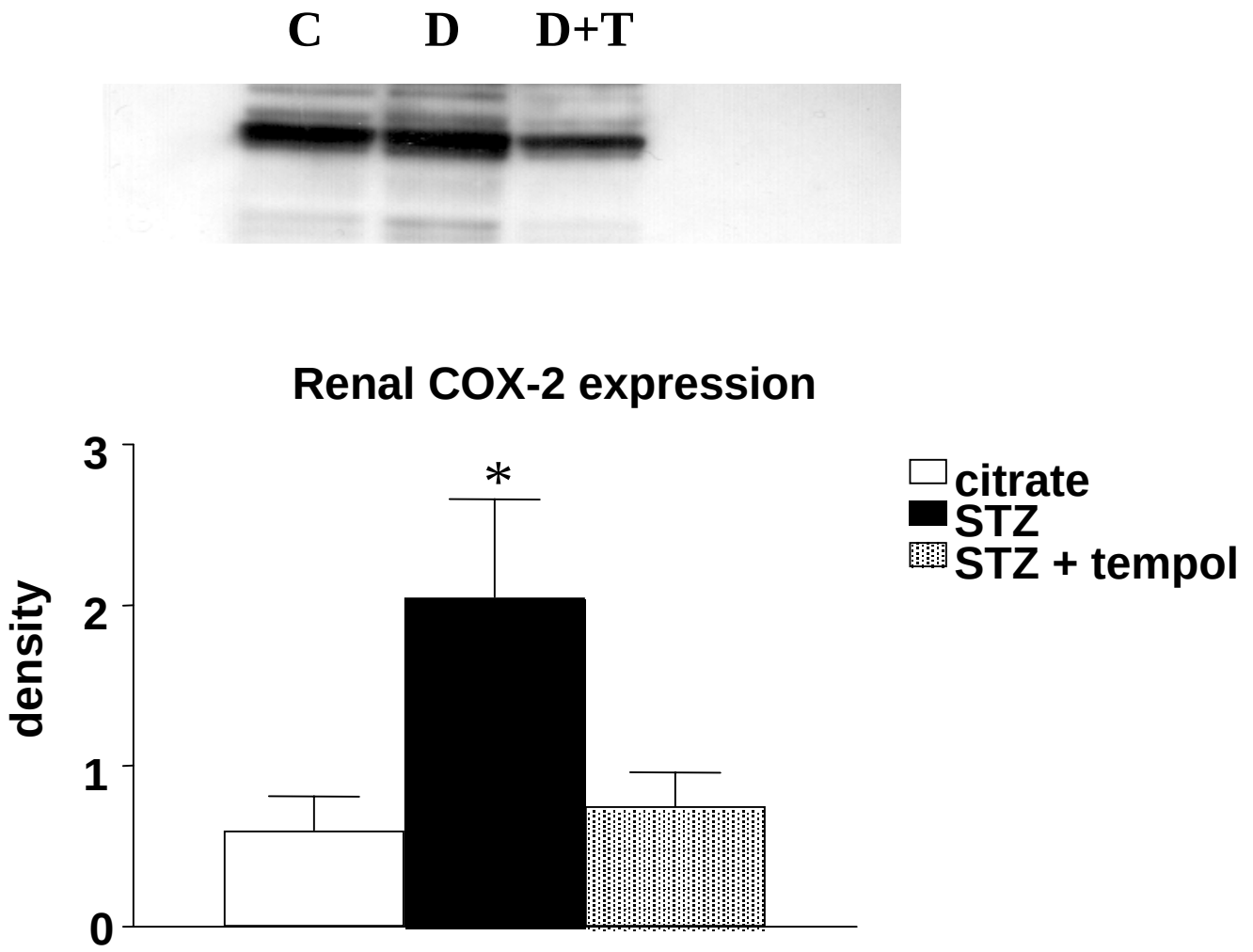

Fig. 4

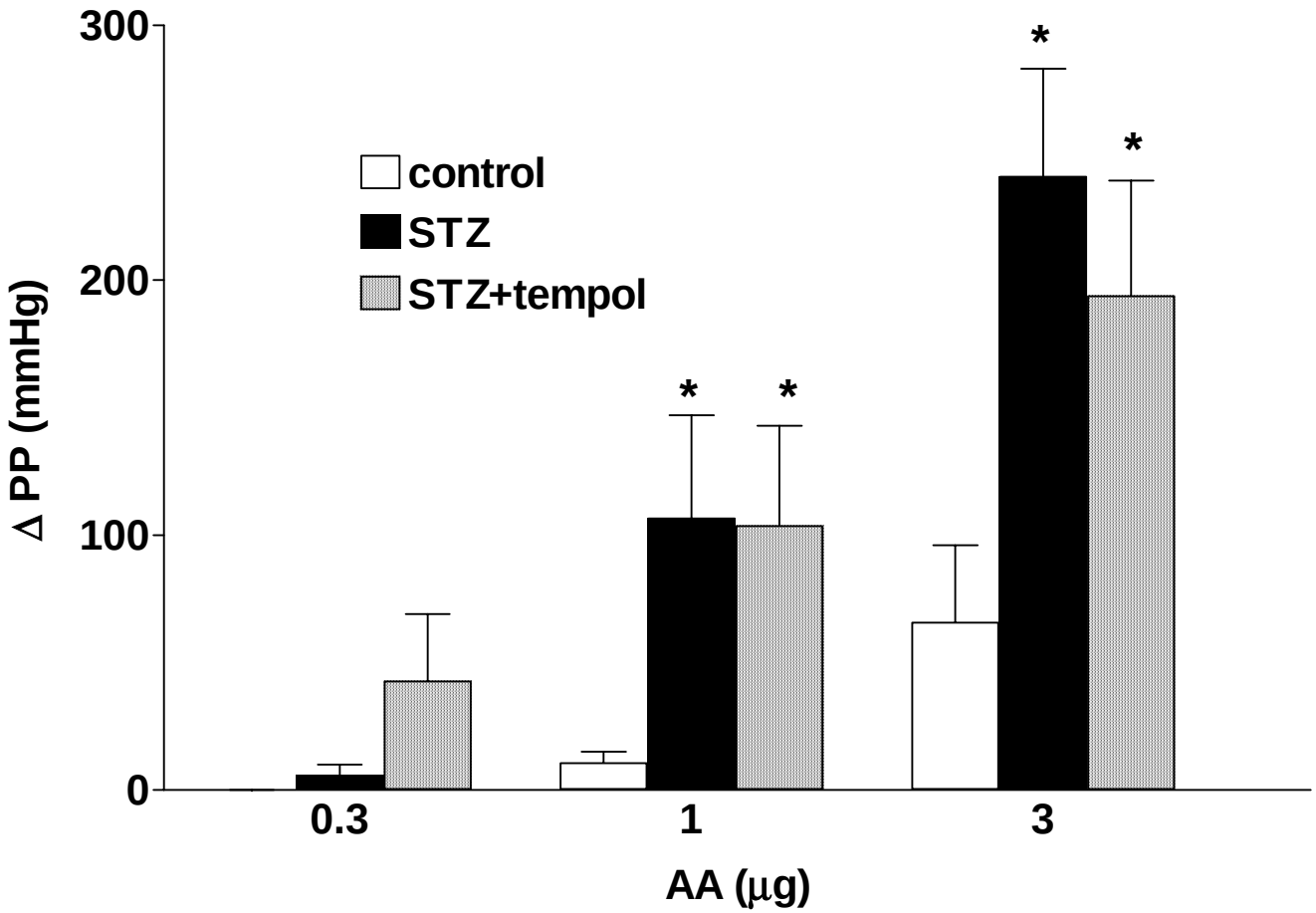

Fig. 5

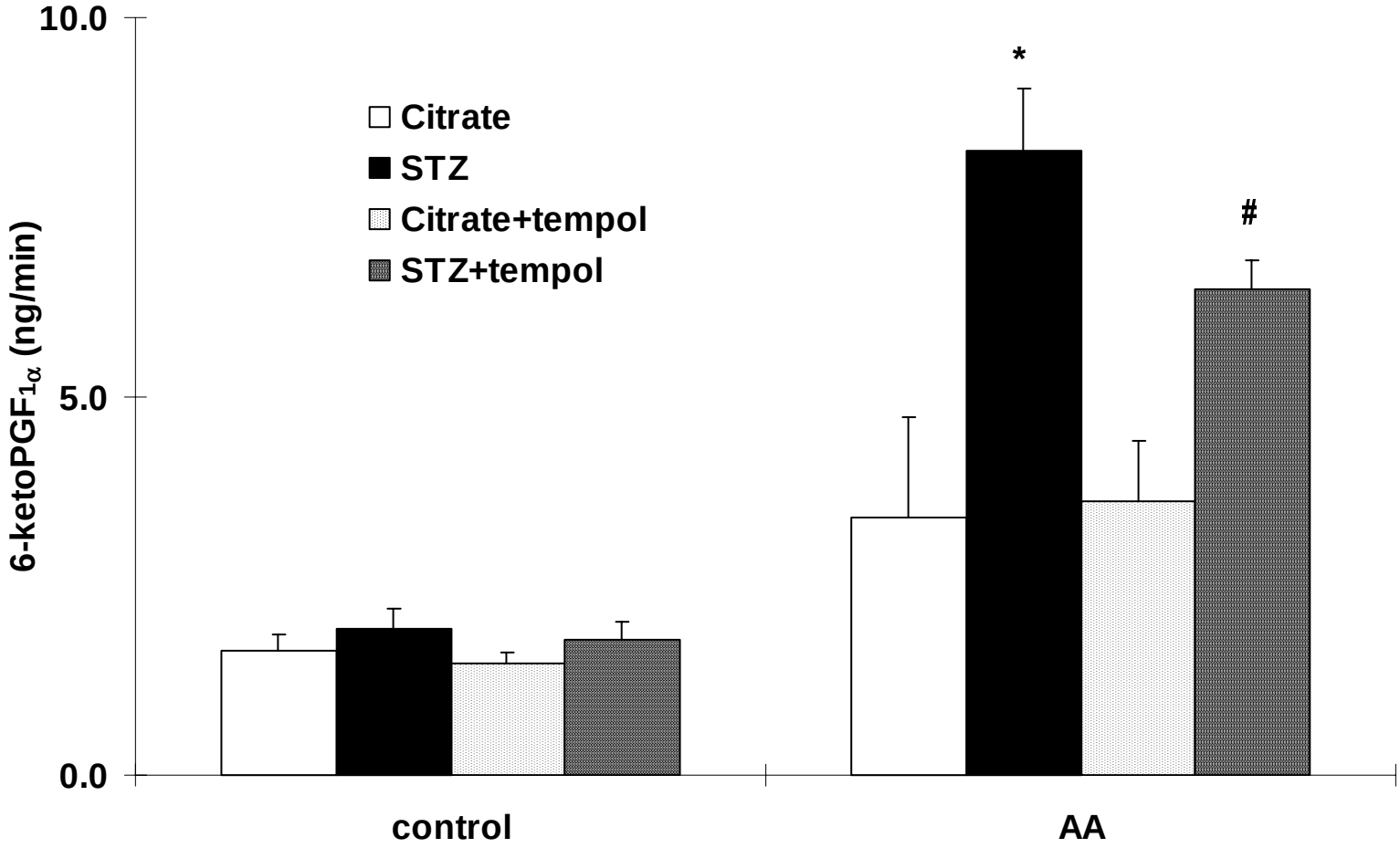

Fig. 6

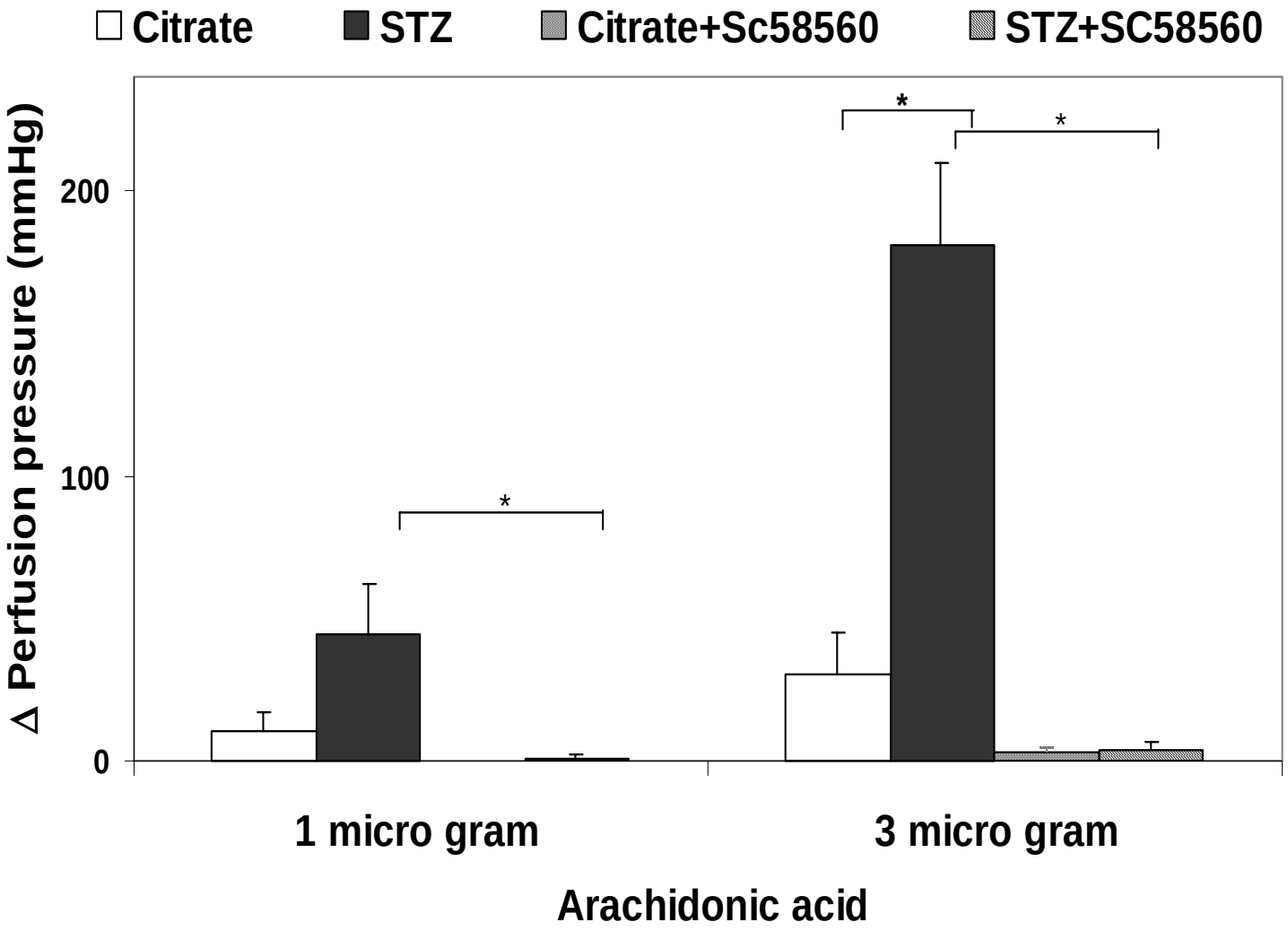

Fig. 7