ABSTRACT

Vasoconstriction within the renal medulla contributes to the development of hypertension. This study investigated the role of reactive oxygen species (ROS) in regulating renal medullary and cortical blood perfusion (MBP and CBP respectively) in both stroke prone spontaneously hypertensive rats (SHRSP) and Wistar rats.

Methods:

CBP and MBP were measured using a laser-Doppler flow meter before and after intrarenal infusion of tempol, the superoxide dismutase (SOD) mimetic or tempol plus catalase, the hydrogen peroxide degrading enzyme.

Results:

Tempol infusion significantly elevated blood perfusion within the renal medulla (MBP) in both SHRSP (by 43 ± 7%, $P < 0.001$) and Wistar rats (by 17 ± 2%, $P < 0.05$) but the magnitude of the increase was significantly greater in the SHRSP ($P < 0.01$). When the enzyme catalase and tempol were co – infused, MBP was again significantly increased in SHRSP (by 57 ± 6 %, $P < 0.001$) and Wistar rats (by 33 ± 6 %, $P < 0.001$), with a significantly greater increase in perfusion being induced in the SHRSP relative to the Wistar rats ($P < 0.01$). Notably, this increase was significantly greater than in those animals infused with tempol alone ($P < 0.01$).

Conclusion:

These results suggest that ROS play a proportionally greater role in reducing renal vascular compliance, particularly within the renal medulla, in normotensive and hypertensive animals, with effects being greater in the

hypertensive animals. This supports the hypothesis that SHRSP renal vasculature might be subjected to elevated level of oxidative stress relative to normotensive animals.

INTRODUCTION

The spontaneously hypertensive rat (SHR) was developed by Okamoto and Aoki (1963) by selectively inbreeding Wistar rats with higher than normal blood pressures to produce a genetic strain where 100% of rats develop hypertension by the age of 10 weeks. The SHRs develop complications associated with hypertension similar to those occurring in the human condition, such as cerebral and myocardial lesions [1].

Hypertension in the SHR model develops as a result of increased peripheral resistance, first produced by neurogenic factors such as enhanced vasoconstrictor PGF2α production [2] and, later, by structural vascular changes associated with increased vascular protein synthesis [3]. In addition, structural changes in the kidneys of SHRs, such as reduced lumen diameter of pre - glomerular arterioles relative to Wistar rats, have also been noted even before hypertension becomes apparent [4]. Notably, fluid retention mechanisms and sodium reabsorption by the kidney are/is also more active in these animals [5]. As such, SHRs are currently one of the best models of human hypertension currently available.

A sub - strain of SHR, which characteristically displays a more severe hypertensive phenotype than the SHR and 80% incidence of stroke by 9 - 13 months of age [3, 6] was subsequently developed and named the stroke - prone SHR rat (SHRSP).

In spite of their chronically elevated blood pressure, SHRSPs have similar renal blood flow and glomerular filtration rates to normotensive Wistar rats, with the result that the SHRSPs have increased vascular resistance relative

to control animals [7]. This is due, at least in part, to structural changes such as vascular remodelling caused by extracellular matrix deposition as well as hypertrophy and impaired vascular endothelium - dependent vasodilatation [8]. These factors increase peripheral resistance and therefore contribute to the increased MAP observed in these animals. The SHR and SHRSP also display increased renal vascular resistance and reactivity to vasoactive substances such as noradrenaline and angiotensin II [9] which also contribute to their hypertensive phenotype.

Acute elevations in arterial blood pressure (BP) markedly increase arteriolar superoxide anion production. This may, in turn, impair endothelial function and set the stage for increased reactivity to vasoconstrictor stimuli and the development of hypertension [10, 11]. Additionally, the SHRSP has higher haematocrit levels and displays increased platelet aggregation when exposed to thrombotic stimuli in comparison to normotensive Wistar rats [12]. Although renal cortical blood flow is the same in both hypertensive and normotensive animals, renal medullary blood pressure (MBP) in SHRSP is lower in comparison to Wistar rats [13]. Notably, this difference is present prior to the development of hypertension and is accompanied by increased vascular tone in the afferent arterioles of juxtaglomerular nephrons [13].

Vascular differences between the SHRSP and Wistar rats, such as increased vascular resistance, may be due to an alteration in levels of circulating reactive oxygen species (ROS). This appears to be the case in SHRs where, for example, the concentration of superoxide anion is elevated in the renal medulla of SHRs compared to normotensive rats [14]. Furthermore, nitric

oxide (NO) production is greater in SHRs compared with normotensive rats [14], which may occur as a compensatory response to mitigate against the vascular effects of elevated concentrations of ROS. However, it is interesting to note that Stankevicius *et al.* [15] demonstrated that the reduced nitric oxide production and reduced vasodilation observed in Goldblatt hypertensive rats was unaffected by the superoxide dismutase mimetic, tempol, suggesting that increased superoxide anion production was not the cause of impaired endothelial function in these animals.

To date, it has not been established if similar molecular mechanisms observed within the SHRs are also at play within the SHRSP. Thus, given the evidence that the renal medulla may be involved in the development of hypertension, and that the availability of ROS may be crucial to its development, the present study sought to determine the role of ROS in mediating regional blood flow within the kidney of SHRSP. This was achieved by locally infusing drugs, which either inhibit or increase the production of reactive oxygen species, into the kidney to determine their effects on renal haemodynamics in SHRSP and normotensive Wistar rats.

METHODS

All procedures were performed in accordance with European Community Directive 2010/63/EU and were approved by University College Cork Animal Experimentation Ethics Committee.

Six groups of male Wistar and SHRSP rats (n= 6-8 rats in each group) 250 - 350 g (~ 12 weeks old), were supplied by Harlan UK Ltd and housed in the Biological Services Unit at University College Cork for at least one week prior to use. The animals were given free access to standard chow (Harland-Teklad, Bicester, UK) and water until 12 hours before surgery when food was restricted. Anaesthesia was induced with a 1 ml bolus dose i.p. of chloralose/urethane (Sigma-Aldrich), 16.5/250 mg / ml respectively, and was maintained using supplemental doses of 0.05 ml given i.v. every 30 minutes. The trachea was cannulated with a short piece (3 - 4 cm) of polypropylene tubing (PP240, internal diameter 1.67mm, external diameter 2.42 mm, Portex Ltd, Harlow, Essex, UK). The tubing was tied into the trachea with thread and the tube cut such that it terminated at nose level to ensure that the animal's normal dead breathing space was not increased. The cannula assisted respiration by providing a patent airway as well as facilitating the removal of any secretions as necessary. The animals were allowed to breathe spontaneously in room air.

The right femoral vein was exposed and cannulated to allow isotonic saline (154 mM NaCl) to be infused at a constant rate of 3 ml / hr and administration

of supplementary anaesthetic. Another cannula was implanted into the right femoral artery and connected to a blood pressure transducer to permit monitoring and recording of blood pressure and heart rate. An interstitial catheter was inserted approximately 4.0 - 5.0 mm into the lower pole of the kidney in order to administer either vehicle or drugs locally into the renal cortico - medullary border (CMB) at a rate of 1 ml / hr. The catheter was then connected onto the end of a 2.5 ml Hamilton glass syringe contained within a mini pump (Model 100, KD scientific, USA) which was set to deliver vehicle or drugs at rate of 16.7 µl / min (1 ml / hr).

Two optical fibre microprobes (MT B500-0 L120, 0.5 mm diameter, Perimed CE 0413, Sweden) were inserted gently into the kidney to depths of 1.5 mm, to measure cortical blood perfusion, and 5.0 mm to measure medullary blood perfusion. The flow probes were connected to a laser-Doppler flow meter (Periflex 4001 Master, Perimed, Sweden) and was pre-calibrated using a PF 1000 calibration device (Perimed, Sweden) that contained, PF 1001 refill motility standard solution. The calibration procedure consisted of taking two measuring points, 0 PU obtained while the probe was on a zeroing disc, and 250 PU when the probe was placed in the motility standard, the PF 1001 motility standard is equivalent to 250 PU, the 100 PU point was set to be equivalent to 1 V.

Following surgery, animals were allowed to stabilise for 90 minutes prior to experimentation.

Upon completion of the experiment animals were euthanised by anaesthetic overdose and the kidney sectioned to confirm the location of the flow probes and cortico - medullary cannula.

Drug Administration

After a post - surgery stabilisation period of 90 minutes, each group of animals received one of three treatments described below:

Control and Tempol Groups

The superoxide dismutase (SOD) mimetic, 4 Hydroxyl-2,2,6,6 tetra-methyl piperidine1-oxyl, obtained from Sigma-Aldrich company Ltd, Switzerland, (tempol; 30 µmol/Kg/min; n = 7 for Wistar rats and 8 for SHRSP), was dissolved in normal saline (0.9 % NaCl) and infused into the CMB at 1 ml / hr**.** Normal saline was used as the vehicle control for experiments and was also infused at 1ml / hr (n = 6 for both Wistar and SHRSP groups).

Tempol plus Catalase Group

Catalase, from bovine liver, (Fluka, Switzerland, 200 IU/Kg/min), an enzyme which degrades H_2O_2 [16], was administered alone for 30 minutes prior to co-administration of tempol (30 µmol/Kg/min) with catalase, also at rate of 1 ml / hr.

Experimental Protocol

Vehicle and tempol protocol: baseline readings for cortical (CBP) and medullary (MBP) blood perfusion, mean arterial pressure (MAP) and heart rate (HR) were obtained over the 16 minute period prior to the start of the renal interstitial infusion. Vehicle (saline) or tempol (30 µmol/kg/min) were infused for 60 minutes, after which a further set of readings were taken over 16 minutes while the infusion continued.

Tempol plus catalase protocol: after the stabilisation period, baseline values were recorded for MAP, HR, CBP, and MBP. Afterward, catalase (200 IU/kg/min) was infused into the renal interstitium alone for 30 minutes, then tempol (30µmol/kg/min) was added to the catalase infusion so that both drugs could be co-infused for a further 60 minutes, after which readings were taken over 16 minutes.

Statistical analysis: Data are presented as mean ± standard error of the mean [17]. The SEM was used as a measure of data dispersion. The statistical significance of any drug-induced changes in the measured parameters was evaluated using Student's paired *t*-tests within the groups. For inter-group comparisons, classical one-way analysis of variance (ANOVA), followed by Tukey's test, was used. Significance was accepted when $P < 0.05$.

RESULTS

Effect of intra-renal infusion of vehicle on CBP and MBP in SHRSPs vs *Wistar rats:*

Initial experiments sought to determine the effect(s) of a 1 ml / hr infusion of saline into the CMB on baseline renal regional blood flow.

Average baseline levels of MAP, HR, CBP, and MBP for all experiments are summarised in table 1. It was noted that MAP in the SHRSPs was significantly higher than that observed in Wistar rats ($P < 0.01$) both before (MAP in SHRSPs = 131 ± 5 mm Hg, MAP in Wistar rats = 102 ± 5 mm Hg) and after the saline infusion (MAP in SHRSP = 134 ± 5 mm Hg, MAP in Wistar rats = 103 ± 6 mm Hg).

In the SHRSPs, renal medullary interstitial infusion of vehicle had no significant effect on either CBP (149 ± 6 PU *vs* 155 ± 6 PU) or MBP (51 ± 4 PU *vs* 51 ± 3 PU; data not shown).

The CBP of SHRSPs was close to the average CBP values in Wistar rats and was also relatively stable over the period of intramedullary infusion of vehicle. However, it is notable that MBP was significantly lower in SHSP versus Wistar rats (51 ± 4 PU *vs* 75 ± 8 PU, $P < 0.05$; figure 1). CBP and MBP in Wistar rats were similarly unaffected by intramedullary infusion of vehicle.

Effect of renal medullary interstitial infusion of tempol on CBP and MBP in SHRSPs vs *Wistar rats:*

The baseline data for MAP, HR, CBP, and MBP are summarised in table 1. Infusion of the SOD mimetic, tempol (30 µmol/kg/min), into the CMB had no significant effect on MAP, HR or CBP in either the SHRSP or Wistar rats. However, intramedullary infusion of tempol significantly increased MBP in both the SHRSPs and Wistar rats, but the magnitude of the increase was significantly greater ($P < 0.01$) in the SHRSPs than the Wistar rats (increased by 43 ± 7 %, $P < 0.001$, in SHRSP and by 17 ± 2 %, $P < 0.05$, in Wistar rats, figure 2)

Effect of renal medullary interstitial infusion of tempol plus catalase on CBP and MBP in SHRSPs vs *Wistar rats:*

In this part of the study we examined whether or not the combined infusion of tempol (30 µmol/kg/min) with the enzyme catalase (200 IU/Kg/min), which inactivates hydrogen peroxide, a vasoactive by-product of tempol metabolism [18], would have effects distinct from those of tempol alone.

Baseline cardiovascular and renal haemodynamic variables in this group of rats were stable over the course of the study, although infusion of tempol plus catalase did evoke a significant increase in HR from 268 ± 8 beats/min before infusion *vs* 282 ± 7 beats/min in SHRSPs ($P < 0.05$). HR was not significantly affected by tempol plus catalase infusion in the Wistar rats. The baseline data are summarised in table 1.

Although intramedullary infusion of tempol in combination with catalase did evoke an increase in CBP in the SHRSPs of some 16 ± 9 %, an effect almost identical to that observed in the Wistar group, this increase was not statistically significant (figure 3).

Once again, as with the intramedullary perfusion of tempol alone in both SHRSPs and Wistar rats, intramedullary infusion of tempol plus catalase evoked a significant elevation in MBP in both strains of rat (increased by 57 ± 6 %; $P < 0.001$, in SHRSPs and by 33 ± 6 %; $P < 0.001$, in Wistar rats) with a proportionally greater increase in MBP being elicited in SHRSPs relative to Wistar rats ($P < 0.01$; figure 3)

When the effects of tempol alone, or catalase addition with tempol, on MBP of the SHRSP were directly compared, we found that MBP was significantly elevated in the tempol plus catalase-infused animals relative to those that received tempol alone ($P < 0.01$, figure 4).

DISCUSSION

There were three important novel findings from the current study. Firstly, we found that MBP was influenced by superoxide anions; secondly, that the production of H_2O_2 also contributed to the level of medullary vascular tone; and thirdly, that the effects of those molecules were greater in hypertensive compared to normotensive animals. In contrast however, the impact of these factors upon CBP was relatively small. It was also apparent that the magnitude of the responses obtained following administration of these compounds was exaggerated in SHRSPs compared to that observed, and previously studied, in normotensive Wistar rats [19, 20], suggesting that the degree of oxidative stress was exaggerated in these animals. These observations support previous findings from our lab of increased carbonylation and sulphanation of key proteins within the medulla of hypertensive rats that is indicative of increase oxidative stress [21, 22].

Control Study

It was evident from the control study group of animals that MBP was lower than CBP in both SHRSP and Wistar rat strains which was consistent with observations from earlier studies from our lab using Wistar rats [19, 20] and, as reported by others, in SHRs [23-26]. Notably however, as medullary perfusion was significantly further reduced in SHRSPs relative to that recorded in the Wistar rats (figure 1), this indicates that renal vascular resistance is elevated in these animals relative to controls, as has been observed previously with this rat strain [13].

The aim of the second part of our study was to investigate how reducing oxidative stress within the kidney, by pharmacologically reducing the basal concentration of superoxide anions, affected cortical and medullary blood flow.

The results of these experiments are discussed in turn below.

Tempol Study

Exaggerated production of superoxide anions by the vascular wall has been observed in different animal models of hypertension including SHRs and SHRSPs. [11, 14, 27-30]. This is significant as superoxide anions and other ROS are potent vasoconstrictors [31], and as such are likely to play a critical role in the pathogenesis of hypertension [32].

Several groups have demonstrated that local or systemic administration of tempol lowers vascular superoxide anion levels and MAP in hypertensive animals [33, 34]. Therefore, in this part of the investigation tempol was administered into the CMB of the study animals in order to inactivate locally produced ROS. We found that by doing so, tempol significantly elevated local blood perfusion in the medullary region. Although not directly comparable because of a different route of drug administration and a different model of hypertension, our findings correlate well with a previous study by Schnackenberg *et al.*, 1998, in which tempol, administered systemically, reduced blood pressure and vascular resistance in SHRs [35]. Similarly, studies by Feng *et al.* (2001) and de Richelieu *et al.*, (2005) demonstrated

that tempol, when added to drinking water, evoked an increase in the medullary blood perfusion without affecting blood pressure. In the latter study, they found that this vasodilatory effect was proportionally greater in SHRs relative to normotensive controls [36, 37]. The reason for the vasodilatory effect of tempol on MBP is likely to be either due to the removal of a direct vasoconstricting effect of ROS on the vasculature, or by an enhanced bioavailability of NO. Indeed, it is feasible that both mechanisms could potentially contribute to the medullary vasodilation witnessed here.Furthermore, tempol also activates BK channels which might further contribute to the increase in medullary perfusion in response to tempol described above [38].

The observation that the increase in MBP following tempol infusion was greater in hypertensive than in normotensive animals supports the suggestion that the kidneys of hypertensive animals are subject to elevated oxidative stress [14]. Significantly however, infusion of tempol locally into the kidney had no effect on MAP in the SHRSP as one might have expected. There may be at least three reasons for this lack of effect; firstly, tempol, when used as an anti - hypertensive agent, is often delivered systemically [39]. However, in the present study the lack of any systemic effects may have been due to the fact that it was administered locally into the kidney. Alternatively, the relatively short period of tempol infusion (60 minutes) may have been insufficient for the evoked increase in medullary perfusion to induce a chronic reduction in MAP (*via* increased fluid mobilisation through the kidneys for example). The third possibility is that although tempol may

well have increased the bioavailability of NO, its vasodilatory effects could have been negated by the tempol - induced formation of H_2O_2, another vasoconstrictor [24].

The fact that tempol had very little effect on CBP in either the hypertensive or normotensive animals suggests either that ROS play an insignificant role in mediating blood perfusion in this region of the kidney or there is another mechanism within renal cortical regions which is able to neutralise or overcome upregulated ROS in the system.

Tempol plus Catalase Study

In addition to the increased ROS generation described in vascular tissues of hypertensive rats discussed previously, a by - product of ROS production, H_2O_2, can also be detected in significant concentrations within vascular tissues of SHRs [40], as well as in the plasma of patients with essential hypertension [41]. However, the role that H_2O_2 plays within vascular tissues, if any, is still not clear. Indeed, it has been suggested that H_2O_2 can act both as vasoconstrictor and a vasodilator depending upon the species, vascular bed and contractile state of the tissues being examined [42-44]. Thus, H_2O_2 can induce dilation of the canine basilar artery, agonist-constricted rat, mouse, and rabbit aortas [45-47] and human, mouse, rat, and rabbit mesenteric arteries [47-51]. But, in contrast, also evokes vasoconstriction of a number of arteries such as the aorta, pulmonary artery, and superior mesenteric arteries of the rat [52-58].

The mechanism(s) underlying H_2O_2 - induced vasoconstriction in these peripheral vessels, such as an increase in cellular Ca^{2+} influx or Ca^{2+} release from intracellular stores of smooth muscle cells, activation of protein phosphorylation enzymes such as phospholipase A2, phospholipase C, protein kinase C, and tyrosine kinase, and stimulation of cyclooxygenase [10, 53, 59], might equally apply to the medullary region of the kidney. Indeed, evidence suggests that H_2O_2 does have important renal actions. For example, when H_2O_2 is elevated within the renal medulla it plays an important role in the development of hypertension and renal injury by directly constricting medullary blood vessels and decreasing sodium excretion, both of which contribute to BP regulation [24, 60]. Furthermore, studies in Sprague Dawley rats have reported that local excessive production of H_2O_2 within the renal medulla of the kidney could evoke hypertension [61].

Thus, the vasoconstrictor actions of superoxide anion and H_2O_2 in the renal medulla would be expected to decrease MBP, reduce sodium excretion, and lead to increased vascular resistance and hypertension [62].

In addition to endogenous H_2O_2, additional H_2O_2 can also be formed as a result of superoxide anion scavenging by tempol [16] when it is infused into the body.

In order to determine if the increase in medullary perfusion, discussed previously when tempol was infused alone, was due to increased availability of NO or to removal of a possible vasoconstrictor action of ROS, tempol was co - infused with catalase. This enzyme should not only remove

endogenously formed H_2O_2 but should also enhance the degradation of H_2O_2 formed as a consequence of tempol action during superoxide removal.

We found that co - administration of the drugs locally into the kidney did indeed result in a marked increase in MBP which was significantly greater than that achieved with tempol alone in the SHRSRs. Moreover, the magnitude of the elevation was greater than that observed in the Wistar rats. These observations imply that firstly, H_2O_2 exerts a major vasoconstrictor effect in the medullary region of the hypertensive rat and that, secondly, H_2O_2 production *via* ROS generation exerts a greater inhibitory effect on medullary perfusion in hypertensive animals relative to controls.

However, in spite of the fact that the drugs were infused locally at the CMB, where they would have acted upon both cortical and medullary regions of the kidney, an increase in perfusion was only observed in the medulla and not the cortex. This suggests that there is a relatively lower generation of ROS, and subsequently H_2O_2, in this region.

Overall conclusions

The results of the present study suggest that the renal vasculature within the medullary region of the kidney of spontaneously hypertensive animals is more sensitive to reactive oxygen species and, consequently, to oxidative stress than normotensive animals.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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