

Title	Effect of tempol and tempol plus catalase on intra-renal haemodynamics in spontaneously hypertensive stroke-prone (SHSP) and Wistar rats
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Publication date	2016-12-09
Original Citation	Ahmeda, A. F., Rae, M. G., Al Otaibi, M. F., Anweigi, L. M. and Johns, E. J. (2016) 'Effect of tempol and tempol plus catalase on intra-renal haemodynamics in spontaneously hypertensive stroke-prone (SHSP) and Wistar rats', Journal of Physiology and Biochemistry, pp. 1-8. First Online. doi: 10.1007/s13105-016-0541-1
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1007/s13105-016-0541-1
Rights	© University of Navarra 2016. Published by Springer Verlag. The final publication is available at Springer via http://dx.doi.org/10.1007/s13105-016-0541-1
Download date	2023-09-30 19:45:49
Item downloaded from	https://hdl.handle.net/10468/3508



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1 **ABSTRACT**

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

7 **Methods:**

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

11 **Results:**

12 Tempol infusion significantly elevated blood perfusion within the renal
13 medulla (MBP) in both SHRSP (by $43 \pm 7\%$, $P < 0.001$) and Wistar rats (by
14 $17 \pm 2\%$, $P < 0.05$) but the magnitude of the increase was significantly
15 greater in the SHRSP ($P < 0.01$). When the enzyme catalase and tempol
16 were co – infused, MBP was again significantly increased in SHRSP (by $57 \pm$
17 6% , $P < 0.001$) and Wistar rats (by $33 \pm 6\%$, $P < 0.001$), with a significantly
18 greater increase in perfusion being induced in the SHRSP relative to the
19 Wistar rats ($P < 0.01$). Notably, this increase was significantly greater than in
20 those animals infused with tempol alone ($P < 0.01$).

21 **Conclusion:**

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

- 1 hypertensive animals. This supports the hypothesis that SHRSP renal
- 2 vasculature might be subjected to elevated level of oxidative stress relative to
- 3 normotensive animals.
- 4

1 INTRODUCTION

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

8 Hypertension in the SHR model develops as a result of increased peripheral
9 resistance, first produced by neurogenic factors such as enhanced
10 vasoconstrictor $PGF2\alpha$ production [2] and, later, by structural vascular
11 changes associated with increased vascular protein synthesis [3]. In addition,
12 structural changes in the kidneys of SHRs, such as reduced lumen diameter
13 of pre - glomerular arterioles relative to Wistar rats, have also been noted
14 even before hypertension becomes apparent [4]. Notably, fluid retention
15 mechanisms and sodium reabsorption by the kidney are/is also more active
16 in these animals [5]. As such, SHRs are currently one of the best models of
17 human hypertension currently available.

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

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

1 to control animals [7]. This is due, at least in part, to structural changes such
2 as vascular remodelling caused by extracellular matrix deposition as well as
3 hypertrophy and impaired vascular endothelium - dependent vasodilatation
4 [8]. These factors increase peripheral resistance and therefore contribute to
5 the increased MAP observed in these animals. The SHR and SHRSP also
6 display increased renal vascular resistance and reactivity to vasoactive
7 substances such as noradrenaline and angiotensin II [9] which also
8 contribute to their hypertensive phenotype.

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

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

1 oxide (NO) production is greater in SHRs compared with normotensive rats
2 [14], which may occur as a compensatory response to mitigate against the
3 vascular effects of elevated concentrations of ROS. However, it is interesting
4 to note that Stankevicius *et al.* [15] demonstrated that the reduced nitric oxide
5 production and reduced vasodilation observed in Goldblatt hypertensive rats
6 was unaffected by the superoxide dismutase mimetic, tempol, suggesting
7 that increased superoxide anion production was not the cause of impaired
8 endothelial function in these animals.

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

18

1 **METHODS**

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

5

6 Six groups of male Wistar and SHRSP rats (n= 6-8 rats in each group) 250 -
7 350 g (~ 12 weeks old), were supplied by Harlan UK Ltd and housed in the
8 Biological Services Unit at University College Cork for at least one week prior
9 to use. The animals were given free access to standard chow (Harland-
10 Teklad, Bicester, UK) and water until 12 hours before surgery when food was
11 restricted. Anaesthesia was induced with a 1 ml bolus dose i.p. of
12 chloralose/urethane (Sigma-Aldrich), 16.5/250 mg / ml respectively, and was
13 maintained using supplemental doses of 0.05 ml given i.v. every 30 minutes.
14 The trachea was cannulated with a short piece (3 - 4 cm) of polypropylene
15 tubing (PP240, internal diameter 1.67mm, external diameter 2.42 mm, Portex
16 Ltd, Harlow, Essex, UK). The tubing was tied into the trachea with thread and
17 the tube cut such that it terminated at nose level to ensure that the animal's
18 normal dead breathing space was not increased. The cannula assisted
19 respiration by providing a patent airway as well as facilitating the removal of
20 any secretions as necessary. The animals were allowed to breathe
21 spontaneously in room air.

22

23 The right femoral vein was exposed and cannulated to allow isotonic saline
24 (154 mM NaCl) to be infused at a constant rate of 3 ml / hr and administration

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

10

11 Two optical fibre microprobes (MT B500-0 L120, 0.5 mm diameter, Perimed
12 CE 0413, Sweden) were inserted gently into the kidney to depths of 1.5 mm,
13 to measure cortical blood perfusion, and 5.0 mm to measure medullary blood
14 perfusion. The flow probes were connected to a laser-Doppler flow meter
15 (Periflex 4001 Master, Perimed, Sweden) and was pre-calibrated using a PF
16 1000 calibration device (Perimed, Sweden) that contained, PF 1001 refill
17 motility standard solution. The calibration procedure consisted of taking two
18 measuring points, 0 PU obtained while the probe was on a zeroing disc, and
19 250 PU when the probe was placed in the motility standard, the PF 1001
20 motility standard is equivalent to 250 PU, the 100 PU point was set to be
21 equivalent to 1 V.

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

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

4

5 **Drug Administration**

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

8

9 ***Control and Tempol Groups***

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

16

17 ***Tempol plus Catalase Group***

18 Catalase, from bovine liver, (Fluka, Switzerland, 200 IU/Kg/min), an enzyme
19 which degrades H₂O₂ [16], was administered alone for 30 minutes prior to co-
20 administration of tempol (30 µmol/Kg/min) with catalase, also at rate of 1 ml /
21 hr.

22

23

1 **Experimental Protocol**

2 Vehicle and tempol protocol: baseline readings for cortical (CBP) and
3 medullary (MBP) blood perfusion, mean arterial pressure (MAP) and heart
4 rate (HR) were obtained over the 16 minute period prior to the start of the
5 renal interstitial infusion. Vehicle (saline) or tempol (30 $\mu\text{mol/kg/min}$) were
6 infused for 60 minutes, after which a further set of readings were taken over
7 16 minutes while the infusion continued.

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

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

21

22

1 **RESULTS**

2

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

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

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

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

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

22

23

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

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

11

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

14 In this part of the study we examined whether or not the combined infusion of
15 tempol (30 $\mu\text{mol/kg/min}$) with the enzyme catalase (200 IU/Kg/min), which
16 inactivates hydrogen peroxide, a vasoactive by-product of tempol metabolism
17 [18], would have effects distinct from those of tempol alone.

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

24

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

5

6 Once again, as with the intramedullary perfusion of tempol alone in both
7 SHRSPs and Wistar rats, intramedullary infusion of tempol plus catalase
8 evoked a significant elevation in MBP in both strains of rat (increased by $57 \pm$
9 6% ; $P < 0.001$, in SHRSPs and by $33 \pm 6 \%$; $P < 0.001$, in Wistar rats) with a
10 proportionally greater increase in MBP being elicited in SHRSPs relative to
11 Wistar rats ($P < 0.01$; figure 3)

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

16

1 **DISCUSSION**

2 There were three important novel findings from the current study. Firstly, we
3 found that MBP was influenced by superoxide anions; secondly, that the
4 production of H₂O₂ also contributed to the level of medullary vascular tone;
5 and thirdly, that the effects of those molecules were greater in hypertensive
6 compared to normotensive animals. In contrast however, the impact of these
7 factors upon CBP was relatively small. It was also apparent that the
8 magnitude of the responses obtained following administration of these
9 compounds was exaggerated in SHRSPs compared to that observed, and
10 previously studied, in normotensive Wistar rats [19, 20], suggesting that the
11 degree of oxidative stress was exaggerated in these animals. These
12 observations support previous findings from our lab of increased
13 carbonylation and sulphanation of key proteins within the medulla of
14 hypertensive rats that is indicative of increase oxidative stress [21, 22].

15

16 **Control Study**

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

1

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

6 The results of these experiments are discussed in turn below.

7

8 **Tempol Study**

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

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

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

12 The observation that the increase in MBP following tempol infusion was
13 greater in hypertensive than in normotensive animals supports the
14 suggestion that the kidneys of hypertensive animals are subject to elevated
15 oxidative stress [14]. Significantly however, infusion of tempol locally into the
16 kidney had no effect on MAP in the SHRSP as one might have expected.
17 There may be at least three reasons for this lack of effect; firstly, tempol,
18 when used as an anti - hypertensive agent, is often delivered systemically
19 [39]. However, in the present study the lack of any systemic effects may have
20 been due to the fact that it was administered locally into the kidney.
21 Alternatively, the relatively short period of tempol infusion (60 minutes) may
22 have been insufficient for the evoked increase in medullary perfusion to
23 induce a chronic reduction in MAP (*via* increased fluid mobilisation through
24 the kidneys for example). The third possibility is that although tempol may

1 well have increased the bioavailability of NO, its vasodilatory effects could
2 have been negated by the tempol - induced formation of H₂O₂, another
3 vasoconstrictor [24].

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

9

10 **Tempol plus Catalase Study**

11 In addition to the increased ROS generation described in vascular tissues of
12 hypertensive rats discussed previously, a by - product of ROS production,
13 H₂O₂, can also be detected in significant concentrations within vascular
14 tissues of SHRs [40], as well as in the plasma of patients with essential
15 hypertension [41]. However, the role that H₂O₂ plays within vascular tissues,
16 if any, is still not clear. Indeed, it has been suggested that H₂O₂ can act both
17 as vasoconstrictor and a vasodilator depending upon the species, vascular
18 bed and contractile state of the tissues being examined [42-44]. Thus, H₂O₂
19 can induce dilation of the canine basilar artery, agonist-constricted rat,
20 mouse, and rabbit aortas [45-47] and human, mouse, rat, and rabbit
21 mesenteric arteries [47-51]. But, in contrast, also evokes vasoconstriction of
22 a number of arteries such as the aorta, pulmonary artery, and superior
23 mesenteric arteries of the rat [52-58].

1 The mechanism(s) underlying H₂O₂ - induced vasoconstriction in these
2 peripheral vessels, such as an increase in cellular Ca²⁺ influx or Ca²⁺ release
3 from intracellular stores of smooth muscle cells, activation of protein
4 phosphorylation enzymes such as phospholipase A₂, phospholipase C,
5 protein kinase C, and tyrosine kinase, and stimulation of cyclooxygenase [10,
6 53, 59], might equally apply to the medullary region of the kidney. Indeed,
7 evidence suggests that H₂O₂ does have important renal actions. For
8 example, when H₂O₂ is elevated within the renal medulla it plays an
9 important role in the development of hypertension and renal injury by directly
10 constricting medullary blood vessels and decreasing sodium excretion, both
11 of which contribute to BP regulation [24, 60]. Furthermore, studies in Sprague
12 Dawley rats have reported that local excessive production of H₂O₂ within the
13 renal medulla of the kidney could evoke hypertension [61].

14 Thus, the vasoconstrictor actions of superoxide anion and H₂O₂ in the renal
15 medulla would be expected to decrease MBP, reduce sodium excretion, and
16 lead to increased vascular resistance and hypertension [62].

17 In addition to endogenous H₂O₂, additional H₂O₂ can also be formed as a
18 result of superoxide anion scavenging by tempol [16] when it is infused into
19 the body.

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

1 endogenously formed H₂O₂ but should also enhance the degradation of H₂O₂
2 formed as a consequence of tempol action during superoxide removal.
3 We found that co - administration of the drugs locally into the kidney did
4 indeed result in a marked increase in MBP which was significantly greater
5 than that achieved with tempol alone in the SHR/SRs. Moreover, the
6 magnitude of the elevation was greater than that observed in the Wistar rats.
7 These observations imply that firstly, H₂O₂ exerts a major vasoconstrictor
8 effect in the medullary region of the hypertensive rat and that, secondly, H₂O₂
9 production *via* ROS generation exerts a greater inhibitory effect on medullary
10 perfusion in hypertensive animals relative to controls.
11 However, in spite of the fact that the drugs were infused locally at the CMB,
12 where they would have acted upon both cortical and medullary regions of the
13 kidney, an increase in perfusion was only observed in the medulla and not
14 the cortex. This suggests that there is a relatively lower generation of ROS,
15 and subsequently H₂O₂, in this region.

16

17 **Overall conclusions**

18

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

23

24

25

1 **CONFLICT OF INTEREST**

2 The authors report no conflicts of interest.

3 **ACKNOWLEDGMENTS**

4 We would like to thank the College of Medicine Research Centre (CMRC),
5 Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia
6 for supporting the research.

7

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