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TEMPOL IMPACTS ON ALTERED METABOLISM AND RENAL VASCULAR RESPONSIVENESS IN THE FRUCTOSE-FED RATS

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Short title: Effects of tempol on renal vasculature in the fructose-fed rats.

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Abstract

This study investigated the effect of tempol (a superoxide dismutase mimetic) on renal vasoconstrictor responses to angiotensin II (Ang II) and adrenergic agonists in fructose-fed model of metabolic syndrome in Sprague-Dawley rats. Rats were fed 20% fructose in drinking water (F) for 8 weeks. One fructose-fed group received tempol (FT) at (1 mmol/l) in drinking water for 8 weeks or as infusion (1.5 mg/kg/min) intrarenally. At the end of treatment regimen, the renal responses to noradrenaline, phenylephrine, methoxamine and angiotensin II were determined. F exhibited hyperinsulinemia, hyperuricemia, hypertriglyceridemia and hypertension. Tempol reduced blood glucose and insulin levels (all $p<0.05$) in FT compared to their untreated counterparts. The vasoconstriction in F to all agonists were lower than control by about 35-65% (all $p<0.05$). FT had higher vasoconstrictor responses to noradrenaline, phenylephrine and methoxamine but not to angiotensin II compared to F by about 41-75% (all $p<0.05$). Acute tempol infusion blunted responses to noradrenaline, methoxamine and angiotensin II in control by 32, 33 and 62% while in F tempol infusion blunted responses to noradrenaline and angiotensin II by 26 and 32% respectively (all $p<0.05$) compared to their untreated counterparts. Superoxide radicals play a crucial role in controlling renal vascular responses to adrenergic agonists in insulin resistant rats. The chronic but not acute tempol enhances the renal vascular responsiveness in the fructose-fed rats.

Key words: Sprague-Dawley rats; tempol; hypertension; angiotensin II; fructose.
Introduction

Reactive oxygen species (ROS), in particular, superoxide radicals were suggested to contribute strongly to the regulation of vascular tone especially during hypertension (Schnackenberg 2002, Vaziri 2004). It is believed that fructose-induced metabolic syndrome is associated with vascular damage due to excess generation of superoxide radical in the vascular system during insulin resistance (Shinozaki et al. 2000, Nandhini et al. 2002). The membrane-permeable superoxide dismutase (SOD) mimetic tempol has been suggested to scavenge superoxide radical. Therefore, it has the ability to reduce oxidative stress and improve insulin sensitivity in the metabolic syndrome (Banday et al. 2005). There is paucity of data regarding the effect of tempol treatment on the development of fructose-induced insulin resistance and hypertension and specifically its effect on vascular function.

Defect in a G protein coupled receptor has been reported in insulin resistance state due to a variety of different reasons. These possibly are hyperinsulinemia, hyperglycemia, dyslipidemia and oxidative stress (Alderson et al. 2003, Trivedi et al. 2004). Therefore, tempol has been utilized to restore normal circulating levels of insulin, glucose, and triglycerides in insulin resistance state (Banday et al. 2005), this can further decrease the oxidative stress and provide a cumulative effect in improving the defective receptor response. ROS are playing a key role in the pathogenesis and maintenance of hypertension due to their effect on renal function and by stimulating salt and water retention (Chabrashvili et al. 2003, Just et al. 2007). Therefore, tempol has been employed to increase sodium excretion, restore normal renal function and blood
pressure (Kopkan et al. 2006, Onuma and Nakanishi 2004). However, the effect of tempol on the renal haemodynamics in fructose-fed rat is less well characterized.

The aim of this study is to gain insight into the effect of superoxide radical scavenging on the renal vascular responses to Ang II and adrenergic agonists in fructose-fed rat using a renal blood flow study. Moreover, this study is designed to examine the effect of tempol on lipid and glucose metabolism, and blood pressure and vascular responsiveness in this model of insulin resistance. We utilized acute and chronic tempol treatment to determine its effects on the metabolic and haemodynamic parameters and on renal vasoconstrictor responses to Ang II and a set of adrenergic agonists. We hypothesized that tempol treatment can restore normal vascular responsiveness to vasoactive stimuli in the fructose-fed rat.

**Materials and Methods**

**Animals.** Male Sprague-Dawley [SD] rats (n=36, 155-185 g) were purchased from the Animal Facility at Universiti Sains Malaysia, Penang, Malaysia. The rats were allowed a period of 3 days to adapt to the new environment (controlled light, temperature and humidity). They were permitted a free access to standard rodent chow (Gold Coin Sdn. Bhd. Penang, Malaysia) (Table 1) and tap water *ad libitum*. The rats were randomly assigned into two groups viz. control (C) which received standard chow and tap water *ad libitum*, and fructose-fed rats (F) which were fed standard chow and 20% (w/v) fructose solution in the drinking water (freshly prepared every day) for 8 weeks *ad libitum*. Rats received tempol at (1 mmol/L) in drinking water (Vazquez-
Prieto et al. 2011, Zhang et al. 2004) for 8 weeks without (T) or with 20% fructose (FT) *ad libitum*. In another part of the experiment, rats received saline (control phase) followed by tempol (1.5 mg/kg/min) acutely as infusion intrarenally at the end of the 8 weeks feeding period. One group of rats (n=4) served as a time control whereby a similar experimental protocol to the actual experiment was used except that tempol was replaced by the vehicle (saline). All the experiments, procedures and handling of animals were approved by the Ethical Committee of Universiti Sains Malaysia through a written agreement [letter ref. USM/PPSF/50 (96) Jld.1].

**Animal surgical preparation.**

*Renal vasoconstrictor responses.* The surgical procedure for the acute renal vasoconstrictor response was adapted from previous studies (Abdulla et al. 2011, Just et al. 2007). The overnight fasted rats were anaesthetized with i.p. injection of sodium pentobarbitone (60 mg/kg, *Nembutal*®, CEVA, Libourne, France). Then, the trachea was cannulated via small cut to permit clear airways. The left jugular vein was cannulated for the infusion of maintenance doses of anaesthetic whenever required. In addition, the left carotid artery was cannulated and the cannula was connected to a fluid filled pressure transducer (model P23 ID Gould, Statham Instruments, UK) linked to data acquisition system (*PowerLab*®, ADInstruments, Sydney, Australia). This allows continuous recording of blood pressure and heart rate (HR). Through a midline incision of the abdomen, the left kidney was exposed and a laser Doppler probe (OxyFlo Probe, Oxford Optronix Ltd., Oxford, UK) was placed in the outermost layer of the renal cortex. The probe was connected to a laser-Doppler flowmeter (ADInstruments, Sydney, Australia) for monitoring of renal cortical blood flow (CBF) using a data acquisition system (*PowerLab*®) throughout the experiment. A cannula was inserted via the left common iliac artery and pushed up until the level
of the renal artery. The tip of the cannula was facing the entrance of the renal artery to inject noradrenaline (NA), phenylephrine (PE), methoxamine (ME) and angiotensin II (Ang II) inside the renal artery. The iliac artery cannula was attached to a second pressure transducer which was also linked to the PowerLab system for recording of renal arterial pressure (RAP). An infusion of saline (NaCl, 9g/l) at a rate of 6 ml/kg/h was started and continued throughout the vasoconstrictor experiment. The urinary bladder was also cannulated to permit free passage of urine. Following completion of surgery, a stabilization period of 1 hour was allowed for animal to equilibrate before commencing to the experimental protocol.

**Measurements.** Calorie intake, body weight, fasting plasma levels of insulin, glucose, triglycerides (TG), cholesterol (Ch), uric acid, SOD-like activity, Ang II and NA in each rat were measured at the end of the 8-week feeding period. The measurement of plasma level of insulin, glucose, TG and Ch is important to assess any dysregulation of the metabolic function in the fructose-fed rats and whether tempol treatment has any impact on these parameters. A blood sample (500µl) was obtained from the carotid artery cannula during the acute study under sodium pentobarbital anaesthesia prior to renal vasoconstrictor experiment. The samples were collected in a pre-cooled eppendorf tubes which were then centrifuged at 3000 rpm. The plasma was carefully withdrawn and immediately stored at -30 ºC for further analysis. The packed cells was gently resuspended in an equivalent volume of saline and replaced back to the animal in order to maintain blood volume as previously suggested (Jirakulsomchok et al. 2012, Najafipour and Ferrell 1995). The plasma level of insulin was measured using a quantitative Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). The plasma levels of glucose, TG, Ch and uric acid were determined using a biochemical auto analyzer (ChemWell®,
Awareness Technology, Inc., Palm City, FL). The kits for these assays were purchased from (BioSystems reagents and instruments, Barcelona, Spain). The plasma SOD-like activity and urinary malondialdehyde (MDA) levels were determined by spectrophotometry using the kit for SOD and MDA assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Ang II was determined by enzyme-linked competitive immunoassay using a commercial Kit (SPI-BIO Bertin Group, Montigny Le Bretonneux, France) by following the procedure recommended by the manufacturer. ELISA kit was used for determination of NA plasma level and was purchased from (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany). Urinary creatinine concentration was determined by spectrophotometry. Adiposity index was determined using the formula \( \text{Adiposity index} = \frac{\text{Retroperitoneal fat (g)} + \text{Epididymal fat (g)}}{\text{Body weight (g)} \times 100} \) as previously reported (Prasad et al. 2010). The renal cortical vascular resistance was estimated using the formula \( \text{Renal cortical vascular resistance (CVR)} = \frac{\text{RAP}}{\text{CBF}} \) (Middlekauff et al. 1997).

**Renal vasoconstrictor response experimental.** In this part of the study, tempol was administered together with fructose in drinking water for 8 weeks. At the end of the treatment period, a single phase acute vasoconstrictor experiment was performed. During that phase, normal saline was continuously infused at 6 ml/kg/h along with the adrenergic agonists and Ang II into the renal artery. Following the stabilization, base line values of SBP, DBP, MAP, RAP, HR and CBF were obtained. Then, vasoconstrictor responses to NA, PE, ME and Ang II were studied by injecting graded doses of these agonists into the renal artery (NA: 25, 50, 100, 200 ng; PE: 0.25, 0.50, 1, 2 µg; ME: 1, 2, 3, 4 µg; Ang II: 2.5, 5, 10, 20 ng) and assessing the renal CBF responses to each dose.
In the second part of the experiment, the renal vasoconstrictor experiment was performed in two distinct phases. Briefly, after the stabilization period, base line values of SBP, DBP, MAP, HR and CBF were recorded. Dose–response curves to NA, PE, ME and Ang II were generated by injecting the above mentioned doses of these agonists into the renal artery and evaluating the vasoconstriction responses to each dose in the presence and absence of tempol. In the first phase (pre-drug), the rats received vehicle (normal saline, NaCl 9g/l) at 6 ml/kg/h along with the adrenergic agonists and Ang II into the renal artery. In the second phase (tempol phase), a dose of 1.5 mg/kg/min of tempol was infused intrarenally (Just et al. 2007). During the second phase, a similar trend of vasoconstrictor injections to the pre-drug phase was followed. The doses of agonists were adapted from previous studies from this laboratory (Abdulla et al. 2009a, 2009b), and were desired to produce a localized effect without any significant impact on the systemic haemodynamic parameters. All drugs were prepared as stock solutions in saline on the day of experiment and stored at +4°C for instant use.

**Chemicals used.** NA (Levophed®, Sanofi Winthrop, London, England), PE (Phenylephrine®, Knoll, Nottingham, UK), ME (Vasoxine®, Calmic Medical Division, Bristol, England) and Ang II (Hypertensin®, CIBA-GEIGY, Basel, Switzerland) were used in the renal vasoconstrictor experiment. Tempol (4-Hydroxy-TEMPO, Sigma-Aldrich, Munich, Germany), a superoxide dismutase mimetic was obtained in powdered form.

**Statistical analysis.** The maximum drop of CBF following injection of adrenergic agonists and Ang II was calculated as the difference between the baseline readings of CBF immediately prior
to agonist injection and the lowest point due to the drop of CBF brought by vasoactive agonists and performed off-line using the software (LabChart 6, ADInstruments, Sydney, Australia). The vasoconstriction produced by adrenergic agonists and Ang II, were taken as the average values triggered by each dose of agonist injected in ascending and descending orders. The mean values for each phase are the overall mean calculated for all doses of each agonist and compared between treated group and the control in the chronic study or between tempol phase and pre-drug phase in the acute study. All data in the current study are presented as mean±SEM. The statistical analysis of the vasoconstriction responses studies (dose-response curves) was done using two-way ANOVA followed by Holm-Sidak’s multiple comparisons post hoc test. The differences between the means of vasoconstriction responses due to all doses of the agonist in all experimental groups was compared using a one-way ANOVA followed by Tukey's multiple comparison post hoc test. The analysis of the differences in the means of the vasoconstrictor response to Ang II in time control group (Fig. 3) was performed using a two-tailed paired student’s $t$-test using GraphPad Prism software (GraphPad® 6.0, San Diego, California, USA). The differences between the means were considered significant at the 5% level.

**Results**

At the end of the feeding period, the calorie intake, the body weight and the plasma glucose, insulin, TG, uric acid, NA and Ang II levels in the F group were higher ($p<0.05$) than C. Tempol interestingly reduced blood glucose and insulin levels (all $p<0.05$) in FT to a value which is nearly approaching C, but did not produce any effect on calorie intake, body weight, TG, uric acid, NA or Ang II in the FT compared to their untreated counterparts (Table 2). Data of plasma Ch level at the end of the study showed similar levels in all the groups. The AUC$_{IVGTT}$ of F was
higher ($p<0.05$) than C and the treatment with tempol had reduced ($p<0.05$) their AUC compared to the untreated counterparts. The adiposity index data showed that the untreated, and tempol treated fructose-fed rats had higher (all $p<0.05$) adiposity index compared to C (Table 2).

The base line haemodynamic parameters during the vasoconstriction experiment of C, F, T and FT groups are shown in Table 3. The base line SBP, DBP and MAP in F were higher ($p<0.05$) than C. However, there was no significant change in the baseline HR, CBF, RAP and CVR of F compared with C. In the tempol treated control, the base line parameters were not different from the untreated rats. The haemodynamic parameters during the pre-drug phase of the two-phase acute vasoconstrictor experiment in the F group were not different from C (Table 4). Moreover, in C or F group, the haemodynamic parameters during acute tempol infusion were not changed compared to their pre-drug corresponding values.

The SOD-like activity in F after the 8 weeks feeding period was lower ($p<0.05$) than C (46±6 vs. 101±10 U/ml) (Fig. 1A). However, upon treating with tempol, FT had higher ($p<0.05$) level of plasma SOD-like activity compared to F (149±18 vs. 46±6 U/ml). Contrariwise, urinary MDA concentration and MDA/creatinine ratio were higher (all $p<0.05$) in F compared to C (7.4±0.5 vs. 3.7±0.3 nmol/ml and 11.4±0.6 vs. 6.8±0.7 nmol/mg, respectively). Tempol treatment for 8 weeks in FT decreased urinary MDA level ($p<0.05$) but not MDA/creatinine ratio (4.3±0.5 vs. 7.4±0.5 nmol/ml) (Fig. 1B,1C).
Renal cortical vasoconstrictor response

Adrenergic agonists. The exogenous NA, PE and ME produced dose-dependent cortical vasoconstrictions in a single phase experiment in C, F, T and FT groups (Fig. 2) or during the two phases of the acute experiment (Fig. 4). The renal vasoconstrictor response to graded doses of NA in F was smaller (all \(p<0.05\)) than C (F vs. C: 50, 100 and 200 ng; 9±1 vs. 27±4, 18±2 vs. 44±4, 34±4 vs. 61±3%). Likewise, the renal vasoconstrictor response to NA in tempol treated control rats at 50 and 100 ng was smaller (\(p<0.05\)) than C (T vs. C: 50, 100 ng; 13±3 vs. 27±5, 27±7 vs. 44±4%). The NA-induced response in FT at the highest dose (200 ng) was greater (\(p<0.05\)) than F (FT vs. F: 200 ng; 46±5 vs. 34±4%) (Fig. 2A). The magnitude of vasoconstriction brought by NA (mean of 4 responses) was smaller (\(p<0.05\)) in F compared to C (F vs. C: 17±2 vs. 38±3%). In addition, there was attenuation (\(p<0.05\)) in the NA-induced responses in the tempol treated rats compared to C (T vs. C: 24±4 vs. 38±3%). The vasoconstriction induced by NA in FT was enhanced (\(p<0.05\)) compared to their untreated counterparts (FT vs. F: 24±3 vs. 17±2%) (Fig. 2B). The renal vasoconstrictor response to graded doses of PE in F was smaller (all \(p<0.05\)) than C (F vs. C: 0.25, 0.5, 1 and 2 µg; 7±1 vs. 23±4, 11±2 vs. 37±4, 22±4 vs. 59±4, 56±6 vs. 75±3%). The PE-induced response in FT at 1 µg was greater (\(p<0.05\)) than F (FT vs. F: 1 µg; 44±7 vs. 22±4%) (Fig. 2C). The overall vasoconstriction produced by PE in F was smaller (\(p<0.05\)) than C (F vs. C: 24±2 vs. 48±2%). Furthermore, the PE-induced response in the tempol treated rats was not significantly different from C. The vasoconstriction induced by PE was increased (\(p<0.05\)) in the tempol treated fructose-fed group compared to the untreated counterparts (FT vs. F: 34±4 vs. 24±2%) (Fig. 2D). The renal vasoconstrictor response to graded doses of ME in F was smaller (all \(p<0.05\)) than C (F vs. C: 1,
2 and 4 µg; 7±1 vs. 22±4, 9±1 vs. 40±5, 29±5 vs. 62±5%). The ME-induced response in FT at the highest dose (4 µg) was greater (p<0.05) than F (FT vs. F: 4 µg; 46±5 vs. 34±4%) (Fig. 2E). The vasoconstriction induced by ME (mean of 4 responses) was smaller (p<0.05) in F compared to C (F vs. C: 12±2 vs. 34±2%). In addition, in the tempol treated rats, the ME-induced response was not different from C. The magnitude of the ME-induced vasoconstriction was raised (p<0.05) in the tempol treated fructose-fed rats compared to F (FT vs. F: 21±3 vs. 12±2%) (Fig. 2F).

In the two phases of the acute experiment, the renal vasoconstrictor response to graded doses of NA in the tempol phase in C was smaller (all p<0.05) compared to the respective pre-drug phase (Tempol vs. pre-drug: C, 25, 50 and 100 ng; 9±2 vs. 24±2, 22±4 vs. 34±3, 36±6 vs. 51±3%) (Fig. 4A). The magnitude of vasoconstriction induced by NA (mean of 4 responses) during the pre-drug but not the tempol phase in F was smaller (p<0.05) than its respective value in C (F vs. C: Pre-drug, 38±3 vs. 47±3; Tempol, 28±4 vs. 32±3%) (Fig. 4B). In addition, the overall vasoconstriction produced by NA in C or F was blunted during the tempol phase compared to the respective pre-drug phase (Tempol vs. pre-drug: C, 32±3 vs. 47±3; F, 28±4 vs. 38±3%, all p<0.05). The PE-induced response during the pre-drug phase in F at 0.5 µg was smaller (p<0.05) than C (F vs. C: Pre-drug, 0.5 µg; 31±5 vs. 52±7%) (Fig. 4C). The vasoconstriction produced by PE during the pre-drug but not the tempol phase was smaller (p<0.05) in F compared to C (F vs. C: Pre-drug, 48±4 vs. 62±4; Tempol, 37±4 vs. 51±4%). Furthermore, in C or F, the magnitude of renal vasoconstriction due to PE was not significantly different during the tempol phase compared to the respective pre-drug phase (Fig. 4D). The ME-induced response during the tempol phase in C at 2 µg was smaller (p<0.05) than its respective response in the per-drug phase.
(Tempol vs. pre-drug: C, 2 µg; 18±4 vs. 53±5%). In addition, the renal vasoconstrictor response to ME at 2 µg during the tempol phase in F was smaller ($p<0.05$) than the pre-drug phase (Tempol vs. pre-drug: F, 2 µg; 26±5 vs. 42±5%) (Fig. 4E). The magnitude of vasoconstriction produced by ME during the pre-drug or the tempol phase in F was not different from its corresponding value in C (Fig. 4F). In addition, the response to ME in the C but not F was reduced ($p<0.05$) during the tempol phase compared to the respective pre-drug phase (Tempol vs. pre-drug: C, 26±4 vs. 39±4; F, 26±4 vs. 37±4%).

**Angiotensin II.**

Exogenous Ang II caused dose-related renal vasoconstrictions in C, F, T and FT groups (Fig. 2G) or in the two phases of the acute experiment (Fig. 4). The Ang II-induced response in F at the highest dose (20 ng) was smaller ($p<0.05$) than C (F vs. C: 20 ng; 35±5 vs. 50±5%). The renal vasoconstrictor response to Ang II in tempol treated control rats at 10 and 20 ng was smaller ($p<0.05$) than C (T vs. C: 10, 20 ng; 6±1 vs. 30±5, 25±5 vs. 50±5%) (Fig. 2G). The magnitude of vasoconstriction due to Ang II (mean of 4 responses) in F was smaller ($p<0.05$) than C (F vs. C: 17±2 vs. 26±2%). Further, the Ang II-induced vasoconstriction in the tempol treated rats was blunted compared to C (T vs. C: 9±2 vs. 26±2%, $p<0.05$). The tempol treatment in F had no impact on their Ang II-induced vasoconstriction compared to the untreated group (Fig. 2H).

In the acute two-phase experiment, there was no significant change in the renal vasoconstriction response to intrarenal Ang II between the first and second saline phases in the time control group (Fig. 3). The renal vasoconstrictor response to graded doses of Ang II in the tempol phase in C
was smaller (all $p<0.05$) compared to the respective pre-drug phase ($\text{Tempol vs. pre-drug: } C, 10$ and $20$ ng; $22\pm4$ vs. $53\pm4$, $30\pm5$ vs. $72\pm3\%$). Moreover, the response to Ang II in tempol treated fructose-fed rats at $10$ ng was smaller ($p<0.05$) than the respective pre-drug phase ($\text{Tempol vs. pre-drug: } F, 10$ ng; $43\pm6$ vs. $53\pm4\%$) (Fig. 4G). The magnitude of vasoconstriction in F produced by Ang II during the pre-drug phase was decreased ($p<0.05$) compared to its respective value in C ($F$ vs. $C$: Pre-drug, $34\pm3$ vs. $47\pm4\%$) (Fig. 4H). Moreover, the Ang II-induced vasoconstriction was reduced during the tempol phase compared to the respective pre-drug phase in C or F group ($\text{Tempol vs. pre-drug: } C, 18\pm2$ vs. $47\pm4$; $F, 23\pm3$ vs. $34\pm3\%$, all $p<0.05$).

Discussion

In the present study, chronic but not acute tempol treatment preserved vascular responsiveness to vasoactive stimuli in addition to its ameliorating effect on insulin resistance and glycemia. At the end of 8 weeks of fructose administration, rats had an increased body weight, hyperinsulinemia, hypertension, hyperglycemia and hyperuricemia. The higher plasma level of uric acid at the end of the 8 weeks is due to insulin resistance or hyperinsulinemia which enhances reabsorption of uric acid in the kidney (Muscelli et al. 1996, Quiñones Galvan et al. 1995). In addition, it was suggested that uric acid clearance is impaired by dyslipidemia or due to a possible effect of fructose on renal transporters (Collantes Estevez et al. 1990, Hu et al. 2009). Tempol had no impact on the plasma uric acid level of the fructose-fed rats. In relation to that, Sanchez-Lozada et al. (2008) demonstrated that tempol could attenuate the adverse effects induced by higher uric acid without affecting its level. The dose of tempol used in the present study was adapted from previous studies and was found to be sufficient to reduce superoxide level in the vasculature.
(Beswick et al. 2001, Elmarakby et al. 2005, Roberts et al. 2009). Fasting blood glucose level in the fructose-fed group at the end of the study was increased considerably in agreement with a previous report after 6 weeks (Mayer et al. 2006) of fructose intake. Tempol improved insulin resistance in the current study and reduced the fasting plasma glucose level significantly. Interestingly, tempol restored plasma insulin to control level indicating a possible increase in insulin sensitivity in the tissues.

The plasma SOD-like activity was smaller but urine MDA level was higher at the end of the feeding period in the fructose-fed group compared with the control. It is suggested that hyperglycemia is playing a role in this decrease in SOD activity through increased lipid peroxidation and superoxide production which result in inactivation or exhaustion of SOD (Hunt and Wolff 1991, Luo et al. 2004). Therefore, reduction in SOD activity is suggested to cause reduced scavenging capacity in fructose-induced insulin resistant rats. The increase in lipid peroxidation in these rats is also evident by the higher MDA levels in urine. Tempol increased the total SOD-like activity in plasma and decreased MDA levels in urine of the fructose-fed rats in the current study. Indeed, tempol was reported to possess a very potent protecting effect against the damaging effects of oxidative stress (Li et al. 2006, Mitchell et al. 1990).

It has been shown that sympathetic activity is enhanced in fructose-induced metabolic syndrome (Farah et al. 2006, Verma et al. 1999). Moreover, fructose intake is also associated with higher renin-angiotensin system (RAS) activity (Kobayashi et al. 1993). The present study showed that the plasma level of NA and Ang II in the fructose-fed rats was remarkably higher than the control. In agreement with that, Tran et al., (2009) showed higher plasma NA and Ang II levels
at the end of 9 weeks of fructose feeding. Tempol treatment did not significantly reduce NA and Ang II levels in the fructose-fed rats compared to the untreated counterparts. This notion is consistent with the lack of any effect of tempol on blood pressure in the fructose-fed rats in the present study. The fructose-fed group had low sensitivity to insulin compared to the control. Tempol had improved insulin resistance and decreased plasma insulin level in the present study and in another model of the metabolic syndrome (Banday et al. 2005).

The current study showed that acute or chronic treatment with tempol resulted in blunted renal cortical vasoconstriction in the control rats. However, in the fructose-fed rats, only chronic treatment increased the renal vascular responses to NA, PE and ME. ROS contribute importantly to the renal vasoconstrictor response to exogenously administered Ang II as well as NA and PE (Just et al. 2007). They played a role in the pathogenesis and maintenance of hypertension via their impact on renal function and also through enhanced salt and fluid retention (Laursen et al. 1997, Rajagopalan et al. 1996). The exact mechanisms behind these effects are not known, however, it is possible that ROS impair endothelium-dependent vasodilation and therefore produce renal vasoconstriction (Rey et al. 2002). This may explain why there is smaller vascular response to adrenergic agonists and Ang II in acute tempol treated control and fructose-fed rats in this study. Our data is consistent with previous studies which showed that superoxide contributes to the acute vascular effects of catecholamines. For example, tempol caused blunted NA-induced vasoconstriction of the rat isolated-perfused kidney (Ozawa et al. 2004) and mesenteric arteries (Somoza et al. 2005). Interestingly, a similar study to what we have utilized showed that tempol acute infusion blunted the renal vasoconstriction responses to Ang II and various G protein-coupled receptor agonists (Just et al. 2007). It has been suggested that
superoxide contributes to the impaired microvascular endothelial function in brain of SOD deficient mice (Faraci et al. 2006). In addition, in SOD-knockout mice, tempol infusion dilated afferent arterioles and reduced the hypersensitivity of these vessels to Ang II (Carlstrom et al. 2010).

Chronic treatment of the fructose-fed rats with tempol resulted in significant enhancement of insulin sensitivity and renal vascular responses to NA, PE and ME. This is well supported by the notion that tempol improves the renal haemodynamics in pathological conditions associated with oxidative stress (Moreno et al. 2008, Nassar et al. 2002). An earlier study in rats with oxidative stress showed that tempol treatment for 3 weeks resulted in normalization of NA constrictor response (Tatchum-Talom and Martin 2004) supporting our suggestion of the involvement of oxidative stress to vascular dysfunction in the renal vasculature. However, tempol treatment blunted the renal vasoconstrictor responses to Ang II in control as well as fructose-fed rats. In an \textit{in vitro} study, tempol resulted in attenuation of Ang II-induced vascular responses in diet-induced insulin resistance (Viswanad et al. 2006). Taken together, the data suggest an inhibitory role of ROS to the vasoconstrictor response to NA in the fructose-fed rats compared to an enhancing effect on NA response in the control rats.

In the current study, we tried for the first time to understand the difference between acute and chronic tempol treatment on renal vascular responses to Ang II and adrenergic stimuli. The concentration of intrarenal tempol was similar to an earlier report in rats which showed similar attenuation of the acute response to Ang II to the one presented in the current study (Dutta et al. 2006). It is evident that acute treatment produced similar effect to chronic treatment in the
control rats. However, in the fructose-fed rats, the acute treatment might not provide sufficient and long lasting free radical scavenging to preserve normal vascular function. This is supported by a previous study which showed that acute tempol treatment although decreased sympathetic activity, but did not cause any significant reduction of superoxide in the blood vessels from DOCA hypertensive rats (Xu et al. 2004).

Chronic treatment of tempol in the present study failed to decrease the elevated level of blood pressure in the fructose-fed rats and this finding agrees with previous reports (Elmarakby et al. 2005, Song et al. 2004, Williams et al. 2004). However, it is in contrary to other studies which showed reduction in blood pressure following tempol administration (Banday et al. 2005, Onuma and Nakanishi 2004). The conflict between these studies is related to different concentrations and routes of administered tempol as well as to the level of blood pressure prior to tempol initiation. As reviewed before (Wilcox and Pearlman 2008), a compensatory mechanism may develop to offset the antihypertensive effect of tempol during its chronic administration such as sodium retention due to tempol-induced increase in H$_2$O$_2$ (Chen et al. 2003). The antioxidant effect of tempol in the current study as well as in others (Wei et al. 2007, Whaley-Connell et al. 2007), could be dissociated from its antihypertensive effect.

This study showed that chronic as well as acute tempol treatment produced no significant effect on base line CBF or systemic blood pressure compared to the untreated counterparts. These findings are supported by previous data which showed no influence of acute or chronic tempol treatment on basal renal blood flow or arterial pressure in normal rats (Schnackenberg and Wilcox 1999). Nonetheless, others have noted that intrarenal infusion of tempol increases blood
flow in the kidney and hypothesized a basal tonic level of ROS production (Chen et al. 2003).

The fact that blood pressure was only assessed under anesthesia in an acute setting and not in awake animals is a limitation of the current study.

In fructose-fed rats, tempol, a SOD mimetic can improve insulin sensitivity, reduce elevated plasma TG and uric acid levels, and preserve the vascular responses to vasoactive stimuli. This indicates that ROS associated with oxidative stress are playing an important role in the development and progression of metabolic, functional and haemodynamic abnormalities in the fructose-induced metabolic syndrome.
Acknowledgements

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Conflict of interest: The authors have no conflict of interest.

References


Table 1: Diet composition derived from nutrients.

<table>
<thead>
<tr>
<th>Composition % weight</th>
<th>Chow*</th>
<th>Chow + fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>--</td>
<td>20% solution in drinking water</td>
</tr>
<tr>
<td>Protein</td>
<td>22%</td>
<td>22%</td>
</tr>
<tr>
<td>Fat</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>Fiber</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Ash</td>
<td>8%</td>
<td>8%</td>
</tr>
<tr>
<td>Calcium</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.8%-1.2%</td>
<td>0.8%-1.2%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.18%-0.24%</td>
<td>0.18%-0.24%</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.0%-1.1%</td>
<td>1.0%-1.1%</td>
</tr>
<tr>
<td>Moisture</td>
<td>13%</td>
<td>13%</td>
</tr>
<tr>
<td><strong>Gross Energy kcal/g</strong></td>
<td>3.3 kcal/g</td>
<td>3.3 kcal/g + 4 kcal/g (fructose)</td>
</tr>
</tbody>
</table>
Table 2: Metabolic parameters of control (C), fructose (F), tempol (T) and fructose fed tempol (FT) treated rats at the end of 8-week feeding period. Values are expressed as mean±SEM of n=6 rats. * P<0.05 vs. C and ‡ P<0.05 of FT vs. F. TG, triglycerides; Ch, cholesterol; SOD, superoxide dismutase; NA, noradrenaline; Ang II, angiotensin II; AUC, area under the curve; IVIGTT, intravenous insulin glucose tolerance test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>C</th>
<th>F</th>
<th>T</th>
<th>FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories intake (Kcal/kg/d)</td>
<td>6</td>
<td>200±5</td>
<td>270±8*</td>
<td>221±7</td>
<td>244±11</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>6</td>
<td>281±7</td>
<td>312±2*</td>
<td>304±6</td>
<td>294±15</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>6</td>
<td>1.44±0.35</td>
<td>7.92±1.39*</td>
<td>1.31±0.20</td>
<td>1.93±0.53‡</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>6</td>
<td>5.2±0.4</td>
<td>8.6±0.8*</td>
<td>5.7±1.0</td>
<td>5.7±0.9‡</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>6</td>
<td>26±3</td>
<td>47±8*</td>
<td>24±3</td>
<td>38±4</td>
</tr>
<tr>
<td>Ch (mg/dl)</td>
<td>6</td>
<td>43±4</td>
<td>35±3</td>
<td>48±7</td>
<td>47±6</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>6</td>
<td>0.48±0.06</td>
<td>0.97±0.14*</td>
<td>0.57±0.11</td>
<td>0.97±0.16*</td>
</tr>
<tr>
<td>Ang II (pg/ml)</td>
<td>6</td>
<td>304±31</td>
<td>510±55*</td>
<td>378±61</td>
<td>381±51</td>
</tr>
<tr>
<td>NA (ng/ml)</td>
<td>6</td>
<td>0.24±0.04</td>
<td>1.37±0.51*</td>
<td>0.39±0.10</td>
<td>0.76±0.14</td>
</tr>
<tr>
<td>AUC_{IVIGTT} (mmol/L.min)</td>
<td>6</td>
<td>142±4</td>
<td>214±7*</td>
<td>124±9</td>
<td>157±7‡</td>
</tr>
<tr>
<td>Adiposity index (%)</td>
<td>6</td>
<td>1.62±0.11</td>
<td>2.87±0.19*</td>
<td>2.06±0.08</td>
<td>2.60±0.21*</td>
</tr>
</tbody>
</table>
Table 3: Renal and systemic haemodynamic parameters measured during the single-phase renal vasoconstrictor experiment in control (C), fructose (F), tempol (T) and fructose fed tempol (FT) treated rats at the end of 8-week feeding period. * P<0.05 vs. C. Bpm; beat per minute, bpu; blood perfusion unit.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>C</th>
<th>F</th>
<th>T</th>
<th>FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>6</td>
<td>123±3</td>
<td>146±6*</td>
<td>128±3</td>
<td>144±2</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>6</td>
<td>91±1</td>
<td>113±7*</td>
<td>100±3</td>
<td>111±3</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>6</td>
<td>112±4</td>
<td>128±3*</td>
<td>115±3</td>
<td>127±3</td>
</tr>
<tr>
<td>RAP (mmHg)</td>
<td>6</td>
<td>109±3</td>
<td>126±6</td>
<td>109±3</td>
<td>124±4</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>6</td>
<td>277±11</td>
<td>310±3</td>
<td>319±13</td>
<td>328±10</td>
</tr>
<tr>
<td>CBF (bpu/min)</td>
<td>6</td>
<td>179±10</td>
<td>185±10</td>
<td>201±7</td>
<td>232±21</td>
</tr>
<tr>
<td>CVR (mmHg/bpu/min)</td>
<td>6</td>
<td>0.62±0.04</td>
<td>0.68±0.06</td>
<td>0.55±0.03</td>
<td>0.62±0.04</td>
</tr>
</tbody>
</table>
Table 4: Renal and systemic haemodynamic parameters measured during the two-phase renal vasoconstrictor experiment in control (C) and fructose (F) fed rats at the end of 8-week feeding period. Bpm; beat per minute, bpu; blood perfusion unit.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>n</th>
<th>Pre-drug Phase</th>
<th>Tempol Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>C</td>
<td>6</td>
<td>131±6</td>
<td>119±5</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>141±6</td>
<td>133±11</td>
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<tr>
<td>DBP (mmHg)</td>
<td>C</td>
<td>6</td>
<td>99±7</td>
<td>87±5</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>103±8</td>
<td>102±9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>C</td>
<td>6</td>
<td>115±6</td>
<td>104±5</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>121±7</td>
<td>118±10</td>
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<tr>
<td>RAP (mmHg)</td>
<td>C</td>
<td>6</td>
<td>114±7</td>
<td>104±6</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>115±7</td>
<td>113±10</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>C</td>
<td>6</td>
<td>302±20</td>
<td>238±10</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>296±21</td>
<td>253±16</td>
</tr>
<tr>
<td>CBF (bpu/min)</td>
<td>C</td>
<td>6</td>
<td>163±5</td>
<td>166±11</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>175±13</td>
<td>136±11</td>
</tr>
<tr>
<td>CVR (mmHg/bpu/min)</td>
<td>C</td>
<td>6</td>
<td>0.71±0.06</td>
<td>0.64±0.03</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>0.68±0.08</td>
<td>0.84±0.05</td>
</tr>
</tbody>
</table>
Figure legends

**Fig. 1:** Plasma superoxide dismutase enzyme (SOD) concentration [A], urine malondialdehyde (MDA) concentration [B] and urine MDA to creatinine ratio in control (C), fructose-fed (F), tempol (T) and fructose-fed tempol (FT) treated rats. Values are expressed as mean±SEM of n=9 rats in each group. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons post hoc test. * p <0.05 vs. C and □ p <0.05 between FT and F.

**Fig. 2:** Dose-response curve and the overall vasoconstriction response produced by noradrenaline [A, B], phenylephrine [C, D], methoxamine [E, F] and angiotensin II [G, H] in control (C), fructose-fed (F), tempol (T) and fructose-fed tempol (FT) groups. Values are mean±SEM of n=6 rats in each group. Data for the dose-response curves [A, C, E and G] were analyzed using two-way ANOVA followed by Holm-Sidak’s multiple comparisons post hoc test. The comparison of the overall vasoconstriction response (mean of 4 responses) due to each agonist [B, D, F and H] between experimental groups was done using a one-way ANOVA followed by Tukey’s multiple comparison post hoc test. * p <0.05 F vs. C, + p <0.05 T vs. C and □ p <0.05 between FT and F.

**Fig. 3:** Dose-response curve and the overall mean (inset) of the renal vasoconstrictor responses to graded doses of angiotensin II in time control group. Values are mean±SEM of n=4 rats. The overall vasoconstriction response (mean of 4 responses) of angiotensin II was compared between the first and second saline phases. Data were analyzed using two-tailed paired Student’s t-test.
Fig. 4: Dose-response curve and the overall vasoconstriction response produced by noradrenaline [A, B], phenylephrine [C, D], methoxamine [E, F] and angiotensin II [G, H] in control (C) and fructose-fed (F) rats. Values are mean±SEM of n=6 rats in each group. Data for the dose-response curves [A, C, E and G] were analyzed using two-way ANOVA followed by Holm-Sidak’s multiple comparisons post hoc test. The comparison of the overall vasoconstriction response (mean of 4 responses) for each agonist [B, D, F and H] was compared between experimental groups using a one-way ANOVA followed by Tukey's multiple comparison post hoc test. * p <0.05 F vs. C (pre-drug) and +, □ p <0.05 tempol vs. pre-drug in C and F respectively.
Fig. 1:

A

Plasma SOD (U/ml)

C  F  T  FT

B

Urine MDA (nmol/ml)

C  F  T  FT

C

Urine MDA/Creatinine (nmol/mg)

C  F  T  FT

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Fig. 2:
Fig. 3:

Angiotensin II (ng)

% Drop in CBF

-90 -80 -70 -60 -50 -40 -30 -20 -10 0 2.5 5 10 20

- First saline phase
- Second saline phase

Angiotensin II
Mean of 4 responses

% Drop in CBF

Saline 1 Saline 2
Fig. 4:

A Noradrenaline (ng)

B Noradrenaline
Mean of 4 responses

C Phenylephrine ($\mu$g)

D Phenylephrine
Mean of 4 responses

E Methoxamine ($\mu$g)

F Methoxamine
Mean of 4 responses

G Angiotensin II (ng)

H Angiotensin II
Mean of 4 responses