CHRONIC TEMPOL TREATMENT ATTENUATES THE RENAL HEMODYNAMIC EFFECTS INDUCED BY A HEME OXYGENASE INHIBITOR IN STREPTOZOTOCIN DIABETIC RATS.

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Running Head: Heme oxygenase inhibition in diabetes.

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ABSTRACT

Heme oxygenase-1 (HO-1) is induced by oxidative stress and plays an important role in protecting the kidney from oxidant-mediated damage in the streptozotocin (STZ) rat model of type-1 diabetes mellitus (DM-1). HO derived metabolites, presumably carbon monoxide (CO), mediate vasodilatory influences in the renal circulation, particularly in conditions linked to elevated HO-1 protein expression or diminished Nitric oxide (NO) levels. We tested the hypothesis that diabetes increases oxidative stress, and induces HO-1 protein expression which contributes to regulate renal hemodynamics in conditions of low NO bioavailability.

Two weeks after the induction of diabetes with STZ (65 mg/kg i.v.), Sprague Dawley rats exhibited higher renal HO-1 protein expression, hyperglycemia, and elevated renal nitrotyrosine levels than control normoglycemic animals. In anesthetized diabetic rats, renal vascular resistance (RVR) was increased, and in vivo cortical NO levels were reduced (p<0.05) compared with control animals. Acute administration of the HO inhibitor Stannous mesoporphyrin (SnMP, 40 µmol/kg i.v.) did not alter renal hemodynamics in control rats, but greatly decreased glomerular filtration rate and renal blood flow, markedly increasing RVR in hyperglycemic diabetic rats. Chronic oral treatment with the superoxide dismutase mimetic Tempol prevented the elevation of nitrotyrosine, the HO-1 protein induction, and the increases in RVR induced by SnMP in the diabetic group, without altering basal NO concentrations or RVR. Increasing concentrations of a CO donor (CORM-A1) on pressurized renal interlobar arteries elicited a comparable relaxation in vessels taken from control or diabetic animals. These results suggest that oxidative stress-induced HO-1 exerts vasodilatory actions that partially maintain renal hemodynamics in uncontrolled DM-1.
Keywords: Heme oxygenase, oxidative stress, nitrotyrosine, Type-1 diabetes, Tempol, Streptozotocin, Nitric Oxide, Renal Hemodynamics.
INTRODUCTION

Heme oxygenases (HO-1 and HO-2) catalyze the conversion of heme to carbon monoxide (CO), free iron and biliverdin (BV) and are both expressed in renal vascular and tubular structures (1,6,10). HO-2 is the constitutive isoform that accounts for the bulk of renal HO activity in normal conditions (10). HO-1 operates as an inducible enzyme with low or undetectable renal levels in the healthy kidney, but markedly increased in several models of renal injury including the streptozotocin (STZ) induced model of DM-1 (1,2,13,18,30).

The heme-HO system also plays a role in regulating vascular function and renal hemodynamics. Metalloporphyrins, which inhibit endogenous HO activity, promote renal vasoconstriction in situations that feature high HO activity, usually due to induced HO-1 (2,6,21,23,32). Moreover, HO-1 upregulation counterbalances the constrictor influences of vasoconstrictors (16,21) or attenuates renal autoregulatory responses to increases in perfusion pressure (6).

Previous reports have established that nitric oxide (NO) synthesis inhibition increases renal HO activity (28) and enhances the functional relevance of the heme-HO system, by intensifying the CO-induced vasodilation (29) and also the vasoconstriction that accompanies the reduction of NO levels (5,29). Therefore, the actions of the heme-HO system could be particularly relevant to influence renal hemodynamics in DM-1, a condition that combines overexpression of HO-1 isoform (13,18,30) and deficient synthesis or bioavailability of NO (15,17,24-26).

Oxidative stress, which is thought to play an important role in the development of diabetic renal complications (25), occurs in renal tissues even at the early stage of diabetes (18, 24,25). The interaction between the heme-HO system and oxidative stress...
in regulating vascular function is not totally understood. CO causes vasodilation (5,29,37), while BV and Bilirubin from HO are believed to mediate protective actions in several models of oxidant induced damage (1,11,12,36). On the other hand, it has also been shown that in some conditions CO can paradoxically induce vasoconstriction, related to enhance reactive oxygen species (20). Therefore, the effect of the HO system induction on renal function in situations of increased endogenous oxidative levels (such as the diabetic kidney) merits further investigation.

The present study tested the hypothesis that the endogenous heme-HO system plays a role in regulating renal hemodynamics in severely hyperglycemic diabetic rats, a condition that combines deficient bioavailability of NO and overexpression of renal HO-1. In order to test it, we studied the expression of HO proteins, the renal nitrotyrosine content (an index of peroxynitrite formation), the in vivo renal cortical NO levels, and the renal vascular responses induced by a HO inhibitor, in control and diabetic rats with or without pretreatment with tempol to reduce oxidative stress.
MATERIALS AND METHODS

Animals

Experiments were performed on anesthetized male Sprague-Dawley rats (275–300 g) housed in temperature controlled environment, with 12:12-h light-dark cycle in the Animal Care Facility at the University of Murcia. All procedures performed were in accordance with the recommendations, from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals, approved by the American Physiological Society. Animals had access to a rodent standard chow and tap water ad libitum throughout the study.

Induction of diabetes

On day one rats were maintained under isofluorane anesthesia and treated with an intravenous single dose of the specific toxic for Beta cells, Streptozotocin (STZ, 65 mg/Kg of body weight) via the femoral vein. Age-matched rats were infused with vehicle (sodium citrate buffer 0,1 mol/l; pH=4.5). After administration of the drug or vehicle, the catheter was removed, the vein was tied off, and the incision was sutured. Once the animals recovered from anesthesia, they were individually housed in cages for the 14 days preceding the beginning of the experiments. Food and water intake was measured periodically throughout the study.

Blood glucose concentration was monitored with a blood glucose meter Accucheck system (Bayer Inc.) in blood samples obtained from the tail vein. To check on the diabetic status, seven days after STZ injection, diabetic animals had higher blood glucose concentration (320±25 mg/dL; n=18) than vehicle treated control rats (70±4 mg/dL; n=18) after 18 hours of fasting.
To address the role of nitrosative stress in these animals, after 48 hours of vehicle or STZ injection, control or STZ diabetic animals received a chronic treatment with the superoxide dismutase mimetic 4-hydroxy-tempo (tempol 1mmol/L, in drinking water) for 12 days (9). Since water intake was higher in diabetic than in control animals, in this group the tempol concentration in the drinking water was adjusted to match the dose in control animals (33.5 ± 0.7 mg/day, n=11 in control rats and 34.5± 0.3 mg/day, n=14 in diabetic animals). Tempol treatment did not alter blood glucose concentration in diabetic (298±45 mg/dL; n=16) or control animals (298±45 mg/dL; n=16), measured on day seven, after 18 hours of fasting.

**Chemicals and solutions:**

Streptozotocin (Sigma) was dissolved freshly in sodium citrate buffer (pH= 4.5). Stannous mesoporphyrin (SnMP), Frontier Scientific (Logan UT), an inhibitor of HO activity (27-29), was dissolved in 50 mmol/l Na₂CO₃. Tempol and other chemicals were obtained from Sigma –Aldrich (Spain). The soluble CO releasing compound CORM-A1 was synthesized as previously described (31) and solubilized in PSS for the vessel reactivity study.

**Experimental Design**

Protocol to contrast the status of expression levels of the HO isoforms and renal nitrotyrosine content, in control and STZ diabetic animals with or without chronic Tempol treatment. After two weeks, kidneys obtained from control or diabetic animals, pretreated or not with Tempol, were initially washed with ice cold PBS buffer, carefully dried and frozen at -80 °C. Whole kidney was homogenized in ice-cold 50-mmol/L Tris-HCl buffer, pH 7.4, containing 1% NP-40, 0.25% sodium deoxycholate, 1-mmol/L EDTA, and 10% protease inhibitor cocktail (Sigma Chemical Co). Kidney
homogenates were then centrifuged (10,000g for 30 minutes) and the supernatant saved for protein assay and Western blot analysis of the HO isoforms or renal nitrotyrosine content.

**Western Blot Analysis of HO-1 and HO-2 isoforms.** Western blot analysis of protein expression was performed as we have previously described (28,33). Briefly, cell-free homogenates of kidney preparations (30 µg protein) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with 5% milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (TBST) buffer at 4°C overnight. After washing with TBST, the membranes were incubated with 1:1000 dilution of rabbit anti-rat HO-1 monoclonal or HO-2 polyclonal antibodies (Stressgen Biotechnologies, Victoria, BC). Chemiluminescence detection was performed using the Amersham ECL detection kit according to the manufacturer's instructions. Protein expression changes were quantified by densitometry analysis and presented as the ratio of HO proteins to ß-actin expression.

**Renal nitrotyrosine content:** Because NO and O$_2^-$ react to form peroxynitrite, the actions of which include nitration of tyrosine residues on proteins, increased renal protein tyrosine nitration is viewed as an indicator of peroxynitrite formation (9,25). Extracted proteins from whole kidney homogenates (700 µg) obtained from control, or diabetic groups, with or without Tempol treatment, were incubated in microtiter wells coated with antibodies recognizing nitrotyrosine and analyzed for the quantitative measurement of nitrosylated proteins by using the Nitrotyrosine ELISA test kit (Hycult biotechnology; Uden, The Netherlands).
Protocol to study the effects of the HO inhibitor SnMP on renal hemodynamics in control and STZ diabetic animals with or without chronic Tempol treatment.

Control or diabetic animals, with and without Tempol treatment, were prepared to study the effects of the HO inhibitor SnMP on renal hemodynamics as previously described (29,33). Briefly, on day 14, animals were anesthetized with ketamine (50mg/kg) and thiobutabarbital (60 mg/kg, ip) and placed on a thermostatically controlled table to maintain body temperature at 37°C. Polyethylene cannulas were placed in the trachea (PE-205) to aid ventilation, the bladder (PE-60) for urine collection, the left femoral vein (PE-50) for administration of fluid and drugs, and the left femoral artery (PE-50) for blood sampling and for the measurement of mean arterial pressure. Next, the left kidney was exposed and a flow probe was placed around the left renal artery for measurement of renal blood flow using a transit time flowmeter (T206, Transonic System Inc.). After completion of the instrumentation procedures, an infusion of 0.15 mol/L NaCl (1,5 mL /h/ 100 g of body weight) containing $[^3]$H inulin (1 μCi/ml) was initiated and maintained throughout the experiment for measurement of glomerular filtration rate (GFR) and for calculations of filtration fraction. Experiments began after a 60 min equilibration period.

Mean arterial pressure and renal hemodynamics values were collected over a 30 min control period immediately before the administration of SnMP (40 µmol/kg, iv) to animals control (n=11), control plus tempol (n=9), STZ (n=8) or STZ plus Tempol (n=9). Data were collected again over a 30 min period, beginning 45 min after the SnMP infusion.
In addition, a group of STZ treated animals (n=8) received the SnMP vehicle alone (Na$_2$CO$_3$, 50 mM).

**Measurement of cortical NO concentration in control and STZ diabetic animals, with or without chronic Tempol treatment, before and after infusion of SnMP.**

Animals treated with vehicle (n=8), Tempol (n= 8), STZ (n=8), or STZ plus Tempol (n=8) were prepared for measurement of NO cortical levels, by using double pulse differential voltammetry, a highly selective and specific technique for in vivo NO measurements previously validated in our laboratory (32,33). Once animals were anesthetized and prepared as described above, the left kidney was exposed by a subcostal flank incision and immobilized in a plastic cup. A NO microsensor was inserted into the renal cortex to a depth of 1-1.5 mm using a micromanipulator.

**Measurement of ex vivo vessel reactivity in interlobar arteries obtained from control and STZ treated animals.**

Exogenous CO can dilate but also constricts renal vessels (20, 29) depending on the redox status (20) or NO levels (29). Furthermore, a reduced vasorelaxant effect of CO has been previously reported in vessels isolated from STZ diabetic animals, due to a reduction in the sensitivity of calcium-activated K+ channels to CO (37). Therefore, we examined whether or not the diabetic status modifies the renal vascular effects of a CO releasing molecule (CORM- A1) (31).

Segments of interlobar artery were microdissected from hemisected kidneys at 4°C and carefully cleared of adherent connective tissue and mounted on glass micropipettes, with monofilament silk, in a perfusion vessel chamber (31). Pressure was gradually increased to 80 mmHg with a pressure-servo unit (Living System...
Instrumentation) and maintained throughout all experiments. The microvessel chamber was placed on the stage of an inverted microscope (Nikon) with attached video camera (CCD). Isolated arteries were continuously superfused with $N\text{-}2$-hydroxyethylpiperazine-$N\text{'}$-2-ethanesulfonic acid (HEPES)-bicarbonate solution containing indomethacin (10 µM). The composition of HEPES solution was (in mmol/L) 130 NaCl, 4 KCl, 1.18 KH$_2$PO$_4$, 1.2 MgSO$_4$, 1.8 CaCl$_2$, 4 NaHCO$_3$, 10 HEPES, 0.03 EDTA, and 6 glucose, pH 7.4.

Pressurized interlobar arteries obtained from control (n=7) and STZ treated animals (n=7) were equilibrated for 1 hour and then preconstricted with the $\alpha$-adrenergic receptor agonist phenylephrine (10 µM). Constricted vessels were allowed to stabilize before a cumulative concentration response curve to the CO donor, CORM-A1, was determined. The change in internal diameters of the interlobar arteries was analyzed using morphometric software (Zeis K300). Vasodilation was calculated as the percent reversal of phenylephrine–induced constriction.

**Calculations and Statistics.**

Results from the clearance study are presented as mean±SE. RBF and GFR are the average of three consecutive 10-minutes observation periods. Data were analyzed by one- or two-way ANOVA with repeated measures followed by the Newman-Keuls post-hoc or the Fisher test. For vessel reactivity studies, a two way ANOVA for repeated measures was used to make comparisons. Other data were analyzed by a Student $t$ test for paired or unpaired observations as appropriate. The null hypothesis was rejected at $P<0.05$. 
RESULTS

General characteristics of control and STZ-infused animals with or without chronic Tempol treatment.

After two weeks of diabetes, baseline characteristics differed significantly between the investigated groups (Table 1). On day 14, diabetic rats showed elevated blood glucose levels, as compared to control animals; tempol had no effects on glucemia. Indeed, hemoglobin A1c (HbA1c), was significantly higher ($p<0.001$) in STZ treated animals ($14 \pm 0.6 \%$; $n=7$) compared to control ($6 \pm 0.5 \%$; $n=10$). Diabetic animals, with or without chronic Tempol treatment, showed the typical features of uncontrolled diabetes: polydipsia, polyphagia and higher kidney/body weight ratio. Body weight was significantly reduced in both groups of diabetic, relative to the control groups of animals.

Renal expression of HO isoforms and renal nitrotyrosine content in control, STZ-infused animals with or without chronic Tempol treatment.

The renal expression of HO-2 protein (relative to $\beta$-actin expression) was similar in control and diabetic animals, with or without Tempol pretreatment (Figure 1). In contrast, diabetes increased HO-1 protein expression after two weeks of STZ injections. Nitrosylated protein concentration in the kidneys from STZ treated animals was higher ($p<0.05$) than that seen in control rats (Figure 2). Chronic tempol administration prevented the increase in HO-1 protein and renal nitrotyrosine content in the diabetic kidneys, so that their levels were comparable to those observed in the control group. Tempol administration had not effects on either HO protein expression or renal nitrotyrosine content in control animals (Figures 1 and 2).
Effects of acute HO inhibition in renal hemodynamics in STZ-infused animals with or without chronic Tempol treatment in STZ induced diabetes.

To determine basal mean arterial pressure and renal hemodynamic values in anesthetized diabetic animals, we pooled the data of the basal period from all STZ rats used in the clearance study given either with Na₂CO₃ or SnMP (Table 2). Relatively to data in control group, diabetic rats displayed similar GFR but decreased mean arterial pressure and renal blood flow, and increased FF. Tempol treatment had not effect on mean arterial pressure or renal hemodynamics in control rats, but it decreased GFR and FF in diabetic animals. Basal urinary volume (in µL/min) was comparable in all experimental groups (55±17, control; 46±9, control + Tempol; 36±4, STZ treated animals; 29±5, STZ + Tempol treated animals).

Figure 3 shows the MAP and renal hemodynamics before and after infusion of SnMP to inhibit HO, in animals from the control, and STZ treated groups with and without Tempol pretreatment. Acute inhibition of HO activity with SnMP had no effect on renal blood flow or RVR in the non diabetic groups, but it greatly reduced (p<0.05) RBF (Δ=-2 ±0.5 ml/min) and increased RVR (Δ=12 ±6 mmHg/ml/min) without altering filtration fraction in untreated diabetic animals. Thus, the renal vasoconstriction induced by the HO inhibitor in diabetes is blunted in animals undergoing concurrent Tempol treatment. In contrast, in those animals given Tempol and STZ, the administration of SnMP reduced GFR but failed to modify RBF (Δ=-0.4 ±0.2 ml/min) or RVR (Δ=1 ±1 mmHg/ml/min) (Figure 3). Accordingly, filtration fraction was significantly reduced by SnMP in diabetic animals treated with Tempol. In the presence of tempol, the SnMP-induced decrease in GFR was similar in control and diabetic rats (Δ=-424±88 µL/min; Δ=-566±207 µL/min, respectively). Although the effect of SnMP on GFR in both Tempol
treated groups tended to be lower than the decrease observed in untreated STZ animals ($\Delta = -878 \pm 206 \mu$L/min), statistical significance was not reached.

Finally, administration of the drug vehicle, $\text{Na}_2\text{CO}_3$, had no effects on blood pressure (from 111±4 to 108±3 mmHg) or renal hemodynamics in diabetic animals: RVR (from 21±2 to 22±1 mmHg/ml/min) and GFR (from 2642±97 to 2357±148 µL/min) before and after vehicle; $p>0.05$ in all instances.

**Effects of the HO inhibitor on the NO concentrations in renal cortex of control and STZ diabetic rats with or without chronic Tempol treatment.**

As figure 4 shows, diabetic and control animals differ in terms of basal in vivo NO bioavailability. Thus, renal cortex concentrations of NO were markedly lower (259±49 nmol/L; $n=10$, $P<0.05$) in STZ diabetic rats compared to corresponding value in control animals (666±92 nmol/L; $n=8$). Unexpectedly, chronic Tempol did not prevent the decrease in cortical NO concentrations in diabetic animals. The SOD mimetic had no effect on NO levels in control rats.

Despite the basal differences in NO levels between groups, acute administration of the HO inhibitor in some additional experiments had no effects on NO cortical levels (nmol/L) in control (from 476±88 to 498±86; $n=4$) or STZ treated animals (from 174±46 to 244 ±46; $n=5$).

**Effects of the CO releasing molecule CORM-A1 on internal diameter of isolated pressurized renal interlobar arteries from control and STZ-treated animals.**

Internal diameter of arteries from control and STZ treated animals was 204±17 µm and 233±21 µm, respectively. Addition of Phenylephrine to the bath elicited a similar reduction in internal diameter in both control (65±6 %) and STZ treated (66±6 %) groups.
Furthermore, as depicted in Figure 5, increasing concentrations of CORM-A1 on isolated pressurized renal interlobar arteries elicited a comparable relaxation in vessels taken from control or diabetic rats.
DISCUSSION

The results from this study demonstrate a significant role of oxidative stress-induced HO-1 in maintaining renal hemodynamics in severe untreated DM-1, a condition that combines high HO-1 protein expression and deficient NO bioavailability.

In anesthetized diabetic rats, we found that the administration of the HO inhibitor SnMP increases renal vascular resistance and lowers renal blood flow in these animals showing high HO-1 protein expression and nitrotyrosine levels, but low cortical NO bioavailability. We also found that the diabetes-induced HO-1 expression, the elevated renal nitrotyrosine, and the reduction in RBF after SnMP, were all prevented in animals chronically pretreated with Tempol to reduce oxidative stress. These changes do not take place in control rats, and the fact that exposure of isolated pressurized renal interlobar arteries to a CO donor elicits similar increases of internal diameter in vessels, from diabetic or control animals, indicates that those differences are not due to an alteration in the vascular response to CO in diabetes. Collectively, these results indicate that the diabetic status creates a setting in which expression of HO-1, and its functional relevance, become essential in maintaining renal hemodynamics.

Our present study demonstrates an increase in HO-1 protein expression in the kidney of severely hyperglycemic rats after two weeks of diabetes. In accordance with previous findings, HO-1 expression was low in the kidneys of control rats (6,10,12,13,28) but high levels of HO-1 were demonstrated in glomeruli (13,18), renal medulla (30) or vascular tissues (37) of animals suffering from STZ induced diabetes. Still, the HO-1 overexpression in this model of DM-1 is not a general finding. Other studies have failed to show HO-1 protein induction in vascular or renal tissues, obtained from insulin treated diabetic animals, or in a more advanced status of diabetes (12,36).
Most likely, differences in the experimental conditions derived from the diabetic state such as the duration of diabetes, levels of oxidative stress, glycemia, or even the presence or the absence of insulin replacement, might explain the discrepancies among different studies. Corresponding to previous observations (36,37), we found no differences in HO-2 protein expression between control and diabetic animals, indicating that HO-1 is the major isoform contributing to the total renal HO activity in diabetic animals, as it was previously demonstrated in conditions of elevated HO-1 protein levels (2,6,10,21).

The current study also provides evidence for oxidative stress as the stimulus for induction of HO-1. Excessive renal or vascular superoxide production, and increased renal nitrotyrosine deposition, have been previously demonstrated in diabetes (15,18,25,36). Increased oxidative stress stimulates the expression of (HO)-1 in several tissues (1), whereas the pharmacological induction of HO-1 has been shown to suppress renal and vascular superoxide levels (11,36). The fact that Tempol normalized the increase in both HO-1 protein expression and nitrotyrosine levels in STZ treated rats suggest a causal link between oxidative stress and upregulation of HO-1 in diabetes, as it has been previously demonstrated (18, 36).

STZ treatment of rats is known to cause severe hyperglycemia, body weight loss, development of renal hypertrophy and later diabetic nephropathy, which are some of the consequences of this experimental model of diabetes similar to the human DM-1. Polyuria, which was observable during the pre-experimental diabetic period in conscious animals, was absent in Inactin-anesthetized animals infused at the same rate of intravenous NaCl (0.9%), due to the fact that in anesthetized animals, volume excretion is mainly determined by the intravenous infusion rate.
The precise mechanisms that govern glomerular hemodynamics in diabetes remain undetermined; multiple vasoactive factors, along with altered renal autoregulatory mechanisms, might be involved. Hyperfiltration is seen in some (4,7,19, 22) but not all (8,14,25,26) studies, depending on the duration, and severity of the model of DM-1. In fact, extremely hyperglycemic rats exhibited reductions in GFR (14,26), and glomerular plasma flow rate, in contrast with the moderately hyperglycemic model (14). In agreement with others (8,19), in the present study, GFR was disproportionately greater than RPF, resulting in elevated FF, which is consistent with increased glomerular pressure in this stage of untreated diabetes.

Hyperfiltration and/or increased RBF is often (4,7), but not always (15,17,25) associated with elevated NO bioavailability, and a number of studies using animals with different glycemic control have produced contradictory findings (17). There is abundant literature suggesting a defective endothelium-dependent NO production or function in diabetes (17,24-26,34,35), which partially involve quenching of NO by superoxide (24,35), likely generated by eNOS uncoupling (34). Our results showing low cortical NO concentrations in hyperglycemic rats confirm those by Palm (26) using a similar electrochemical technique, which detects directly cortical NO concentrations on a real time basis (32,33). Interestingly, the reduced cortical NO levels was attributed to limited availability of the NOS substrate, L-Arginine, (26). Based on these observations, the combination of poor metabolic control, reduced renal NO bioavailability and/or increased oxidative stress levels, might contribute to the observed hemodynamic abnormalities leading to diabetic renal dysfunction. Assuming the complexity of the scenario, and the multiple factors involved, our data demonstrate, for the first time, that
HO activity collaborates to the regulation of renal hemodynamics in the diabetic status, a condition linked to low endogenous NO levels and elevated oxidative stress.

The effects of the antioxidant treatment on renal hemodynamics in diabetes are not fully established. Several in vivo and in vitro studies, using different doses or compounds, found that antioxidant treatments alter renal hemodynamics in diabetes (19), whereas some other studies have found no effect (22, 35). In the present study, we report that Tempol reduced GFR and filtration fraction in diabetic animals. Although we do not have a satisfactory explanation for this effect, we can speculate that Tempol, by reducing the HO-1 induction in diabetic rats, tends to lower renal blood flow and has a significant effect on glomerular filtration. The precise mechanisms involved are unknown, although it seems clear that the fact that tempol did not restore NO levels and reduced HO-1 induction, produces a state of predominant renal vasoconstriction in diabetes, indicating that mechanisms other than oxidative stress are causing the NO deficiency and decreased RBF in diabetic rats. Consequently, we hypothesize that both vasodilator mediators, NO and CO, play a role in the regulation of renal hemodynamic in diabetes, where low NO levels and elevated oxidative stress lead to a situation where renal hemodynamics are compromised and can only partially be maintained by HO-derived products from the induced HO-1.

Since SnMP is a non-specific HO inhibitor, from our data it is not possible to separate the relative contribution of HO-1 and HO-2 isoforms in maintaining renal hemodynamics in diabetes. This may be of importance, because both HO-1 and HO-2 isoforms have been demonstrated to be essential in preventing STZ-induced renal dysfunction and morphologic injury in diabetic mice (12). In the current study, HO-1, but not HO-2 protein is overexpressed in diabetic animals; consequently, the more
pronounced effects of SnMP on RBF and RVR observed in diabetic rats can be attributed to the induction of HO-1. This is further supported by data demonstrating that high levels of renal HO-1 protein expression are followed by increased CO release and a greater vasoconstriction after SnMP (21). Unexpectedly, Tempol caused a reduction in GFR after SnMP administration in control rats. Because the same effects were observed in diabetic animals given Tempol, in which HO-1 protein is not upregulated, it seems likely that chronic Tempol administration modifies the regulation of glomerular filtration in such a way that it becomes dependent on HO-2 activity. The mechanisms underlying this dependence are currently unknown.

That endogenous Heme-HO system contributes to regulate renal hemodynamic function in the diabetic kidney is, at this time, a novel finding. Still, these results are not surprising, based on the demonstrated vasodilatory influences of some HO derived products, i.e. CO, (3, 5, 6, 16, 28). Moreover, our data point out to a minimal influence of endogenous HO activity on renal hemodynamics in basal conditions (5, 23), which may become more obvious under conditions of high oxidative stress in diabetes.

The molecular mechanisms through which inhibition of endogenous HO activity promotes renal vasoconstriction in diabetic animals cannot be obtained directly from this study, but there is good evidence that diminished synthesis of one or more HO products, via inhibition of HO activity, would affect renal hemodynamic function. CO or CO donors increase diameter of renal vessels in vitro (5,31), and renal blood flow and glomerular filtration rate in vivo (3,31). Moreover, inhibition of endogenous HO activity with metalloporphyrins, presumably through CO reduction, causes renal vasoconstriction particularly in settings in which HO-1 expression is increased (2,6,23) or NO synthesis is diminished (5,29). Since SnMP was effective to reduce HO dependent- CO
production and urinary CO excretion (27), the renal effects of SnMP in diabetic animals are likely a result of withdrawal of the vasodilatory actions of CO.

A diminished vasorelaxant effect of CO was demonstrated in diabetes, due to reduction in the sensitivity of calcium-activated K+ channels to CO (37), thus, we examine whether or not the diabetic status modifies the vascular effects of a CO donor. In the current study, renal interlobar arteries from diabetic animals did not differ in terms of vasodilatory responses to CORM-A1 from normal vessels, suggesting that increased CO production, rather than changes in vascular response to CO, could account for the observed *in vivo* effects. Although the interlobar artery is not a typical resistance-sized artery, and the impact of changes in diameter of these arteries on renal vascular resistance could be rather limited, a similar effect of the CO donor in smaller arteries would be expected to significantly affect renal blood flow. Alternatively, and based on previous evidences, CO could also work as a renal vasoconstrictor (20,29) through generation of reactive oxygen species in renal vasculature (20). Moreover, biliverdin and bilirubin can inhibit CO-induced $O_2^-$ production and vasoconstriction (20). In our experimental conditions, the presence of high levels of endogenous HO-1 would generate concurrently CO, BV and BR; thus, we speculate that increased BV and BR production would additionally support the CO induced vasodilation. Assuming that arteries in diabetic animals are not exposed *in vitro* to the same *in vivo* metabolic milieu, the overall data suggests that an increase in CO production, rather than changes in vascular sensitivity to CO, might be operating in renal diabetes.

In addition to the direct effects of CO on the vasculature, CO also opposes agonists-induced vasoconstriction (16), originating a greater influence of endogenous vasoconstrictor systems after HO inhibition (16, 21, 29), particularly in the presence of
L-NAME (29). Therefore, the greater vasoconstrictor response to SnMP in the diabetic animals could also be caused by a greater influence of endogenous vasoconstrictor systems, including free radical species, which are activated in diabetes (7,15,19,25). In this regard, previous work from our laboratory has shown that HO-1 induction preserves renal hemodynamic function by reducing peroxynitrite formation during renal ischemia (33).

In summary, we show that HO-1 expression, along with the renal vasoconstrictor response to HO inhibition, is increased in severe hyperglycemic diabetic rats showing low NO levels. Furthermore, Tempol treatment in diabetic animals, which reduced the oxidative stress and HO-1 protein expression, also blunted the renal vascular response to HO inhibition in diabetic animals. Therefore, high levels of oxidative stimulate HO-1 protein expression, and renal vascular actions mediated by HO in the kidney of severely hyperglycemic diabetic rats, probably due to increased generation of CO and/or bile pigments. The relevance of those findings is emphasized by the fact that heme-HO may be a pharmacologically modifiable system which contributes to renal hemodynamics in poorly controlled diabetes.
GRANTS

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REFERENCES


FIGURE LEGENDS

Figure 1
Assessment of heme oxygenase (HO)-1 and -2 expression by immunoblotting of proteins in rat kidneys from control (n=7); STZ-treated rats (n=7), Tempol-supplemented control (n=4) and STZ- treated rats (n=7). Densitometry data represent the ratio between the density of HO isoforms and β-actin bands in each group. † indicates P<0.05 relative to the corresponding value in control animals. # indicates P<0.05 versus STZ animals without Tempol treatment.

Figure 2
Renal nitrotyrosine content in whole kidney homogenate obtained from control (n=12); STZ-treated rats (n=8); and Tempol-supplemented control (n=9) or STZ-treated animals (n=11). † indicates P<0.05 relative to the corresponding value in control animals. # indicates P<0.05 versus STZ animals without Tempol treatment.

Figure 3
Mean arterial pressure (MAP), renal blood flow (RBF), renal vascular resistance (RVR) and glomerular filtration rate (GFR) before (open bars) and after 45 minutes (closed bars) treatment with stannous mesoporphyrin (SnMP; 40 μmol/Kg, iv) in control (n=11); or STZ-treated rats (n=8), and Tempol-supplemented control (n=9) or STZ-treated animals (n=9). Results are the mean ± SE. * indicates P<0.05 relative to data before SnMP; † indicates P<0.05 relative to the corresponding value in control animals. # indicates P<0.05 versus STZ animals without Tempol treatment.
Figure 4

Panel A) Representative individual voltammetric curves observed in basal conditions in animals treated with vehicle, or STZ. The height of the peak obtained at 650 mV is proportional to the concentration of NO present in renal cortical tissue.

Panel B) NO renal cortical levels (nmol/l) in animals treated with vehicle (n=8), Tempol (n=8), STZ (n=10), or STZ plus Tempol (n=8). Results are the means ± SE. † indicates P<0.05 relative to the corresponding value in control animals.

Figure 5

Concentration dependent vasodilation to the CO releasing molecule, CORM-A1, in isolated pressurized rat interlobar arteries obtained from control (n=7) and STZ treated animals (n=7) after two weeks of injection. The vascular tissues were preconstricted with phenylephrine (10 µM) and vasodilation was expressed as the percent reversal of phenylephrine –induced constriction.
Table 1. *General characteristics of control and diabetic animals with and without Tempol treatment.*

<table>
<thead>
<tr>
<th></th>
<th>CONTROL Without Tempol</th>
<th>CONTROL With Tempol</th>
<th>STZ Without Tempol</th>
<th>STZ With Tempol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>308 ± 8</td>
<td>338 ± 5</td>
<td>250 ± 86 †</td>
<td>254 ± 5 †</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.9 ± 0.0</td>
<td>2.0 ± 0.0</td>
<td>2.2 ± 0.1 †</td>
<td>2.3 ± 0.1 †</td>
</tr>
<tr>
<td>KW /BW ratio (%)</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.9 ± 0.1 †</td>
<td>0.9 ± 0.0 †</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>103 ± 9</td>
<td>111 ± 3</td>
<td>445 ± 27 †</td>
<td>479 ± 33 †</td>
</tr>
<tr>
<td>Water Intake (ml/day)</td>
<td>31 ± 2</td>
<td>39 ± 1</td>
<td>233 ± 18 †</td>
<td>205 ± 6 †</td>
</tr>
<tr>
<td>Food Intake (g/day)</td>
<td>23 ± 2</td>
<td>23 ± 0</td>
<td>40 ± 3 †</td>
<td>39 ± 3 †</td>
</tr>
</tbody>
</table>

*Values are means ± SE; STZ streptozotocin; † P < 0.05 versus corresponding value in control animals.*
Table 2. Baseline of blood pressure and renal hemodynamics parameters in control and diabetic animals with and without Tempol treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CONTROL</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Tempol</td>
<td>With Tempol</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>120 ± 3</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>RBF (ml/min)</td>
<td>7.7 ± 0.3</td>
<td>7.9 ± 0.7</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47 ± 0.6</td>
<td>45 ± 0.7</td>
</tr>
<tr>
<td>RVR (mmHg/ml/min)</td>
<td>16 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>GFR (µL/min)</td>
<td>2327 ± 171</td>
<td>2105 ± 121</td>
</tr>
<tr>
<td>FF</td>
<td>0.30 ± 0.03</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; STZ streptozotocin; MAP, Mean arterial pressure; RBF, renal blood flow; GFR, glomerular filtration rate; RVR, renal vascular resistance; FF, filtration fraction; † indicates $P<0.05$ relative to the corresponding value in control animals; # indicates $P<0.05$ versus animals without Tempol treatment by Two Way ANOVA.
FIGURE 1

A) CONTROL

STZ

HO-1 ▲
HO-2 ▲
β-actin ▲

B) Without With TEMPOL

Without With TEMPOL

HO-2 / actin (ratio)

<table>
<thead>
<tr>
<th></th>
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<th>With</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
<td>0.50</td>
<td></td>
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</tr>
<tr>
<td>0.25</td>
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<tr>
<td>0</td>
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<td></td>
</tr>
</tbody>
</table>

HO-1 / actin (ratio)

<table>
<thead>
<tr>
<th></th>
<th>Without</th>
<th>With</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


g533-actin

Without
With

TEMPOL

†

#
FIGURE 5

% VASODILATION

CORM-A1 (Log M)

CONTROL (n=7)

STZ (n=7)