Metabolic syndrome increases endogenous carbon monoxide production to promote hypertension and endothelial dysfunction in obese Zucker rats

**Short title:** Increased carbon monoxide in metabolic syndrome

Fruzsina K. Johnson,¹ Robert A. Johnson,¹ William Durante,² Keith E. Jackson,¹ Blake K. Stevenson,¹ Kelly J. Peyton²

¹Tulane Hypertension and Renal Center of Excellence, and Department of Physiology, Tulane University Health Sciences Center, New Orleans, LA

²Micheal E. DeBakey VA Medical Center, and Departments of Medicine and Pharmacology, Baylor College of Medicine, Houston, TX

**Corresponding author:** Fruzsina K. Johnson, MD

Department of Physiology
Tulane University Health Sciences Center
1430 Tulane Avenue, SL-39
New Orleans, LA 70112
Tel.: (504) 988-4363
Fax: (504) 988-2675
E-mail: fjohnson@cvlabs.org

Copyright © 2005 by the American Physiological Society.
Abstract

Vascular heme oxygenase (HO) metabolizes heme to form carbon monoxide (CO). Increased heme-derived CO inhibits nitric oxide synthase and can contribute to hypertension via endothelial dysfunction in Dahl salt-sensitive rats. Obese Zucker rats (ZR) are models of metabolic syndrome. This study tests the hypothesis that endogenous CO formation is increased and contributes to hypertension and endothelial dysfunction in obese ZR. Awake obese ZR showed increased respiratory CO excretion which was lowered by HO inhibitor administration (ZnDPBG 25µmol/kg/24hr ip). In awake obese ZR, chronically instrumented with femoral arterial catheters, blood pressure was elevated and was decreased by the HO inhibitor, ZnDPBG. Body weight, blood glucose, HbA1c, plasma insulin, total and LDL cholesterol, oxidized LDL, and triglyceride levels were elevated in obese ZR, and, except for LDL cholesterol, were unchanged by HO inhibition. Total HO-1 protein levels were not different between lean and obese ZR aortas. In vitro experiments used isolated skeletal muscle arterioles with constant pressure and no flow, or constant midpoint, but altered endpoint pressures to establish graded levels of luminal flow. In obese ZR arterioles, responses to acetylcholine and flow were attenuated. Acute in vitro pretreatment with a HO inhibitor, CrMP, enhanced acetylcholine and flow-induced dilation and abolished the differences between groups. Furthermore, exogenous CO prevented the restoration of flow-induced dilation by the HO inhibitor in obese ZR arterioles. These results suggest that HO-derived CO production is increased and promotes hypertension and arteriolar endothelial dysfunction in obese ZR with metabolic syndrome independent of affecting metabolic parameters.

Keywords: rodent models of metabolic syndrome, carbon monoxide, heme oxygenase, blood pressure, endothelial dysfunction
Introduction

Metabolic syndrome is characterized by upper body obesity, insulin resistance, hyperlipidemia, and hypertension, and represents a major and growing health problem in the United States (11). Endothelial dysfunction, due to impaired nitric oxide function, is a key feature promoting microvascular disease in resistance vessels, which contributes to hypertension during obesity (43) and metabolic syndrome. The obese Zucker rat (ZR) is a well established genetic model of obesity (58). Due to a non-functional leptin receptor gene (19) and the consequent hyperphagia, these animals develop obesity and metabolic syndrome including insulin resistance (29), hyperlipidemia (29) and hypertension (13). Previous studies have shown impaired endothelium-derived nitric oxide-dependent vasodilation in various vascular beds of obese ZR (12,13,33,41), including skeletal muscle resistance vessels (12, 13).

Metabolic syndrome represents a major cardiovascular risk factor, which is further enhanced by the increased chance of type 2 diabetes development (11). It is known that patients with type 2 diabetes show increased respiratory carbon monoxide levels (39). While elevated blood glucose levels in obese ZR are not as prominent as some diabetes models, it is possible that the elevated glucose, in conjunction with other obesity risk factors, could promote metabolic and developmental changes to promote carbon monoxide production.

The primary endogenous source of carbon monoxide formation is via enzymatic breakdown of heme by heme oxygenase (47). Numerous tissues (35), including vascular endothelial and smooth muscle cells express, heme oxygenase (8,16,24). Two major enzymatically active isoforms of heme oxygenase (35) are the inducible heme oxygenase-1 and the constitutive heme oxygenase-2. Pathological conditions (35), such as angiotensin II-induced (20), Dahl/Rapp salt-sensitive (21,48), or DOCA-salt hypertension (22) can increase vascular heme oxygenase-1 expression. While heme-derived carbon monoxide can relax vascular smooth
muscle (10, 15, 27) and protect against some aspects of apoptosis (9), it also interferes with the
vasodilator effects of the nitric oxide system (37, 49, 56) and promotes endothelium-dependent
vasoconstriction (23,42).

Since patients with elevated glucose can present with elevated levels of carbon monoxide
production (39) and since carbon monoxide can inhibit nitric oxide synthase (37,49,56) and
promote hypertension, we hypothesized that heme-derived carbon monoxide formation is
increased and contributes to hypertension and arteriolar endothelial dysfunction in obese ZR. To
test this hypothesis we used awake lean and obese ZR rats and examined the effects of an
inhibitor of heme oxygenase on blood pressure and carbon monoxide production. To examine
endothelial function, we also conducted experiments using skeletal muscle arterioles taken from
age matched lean and obese ZR, and examined the responses to an endothelium-dependent
vasodilator and increases in luminal flow while in the presence or absence of an inhibitor of
heme oxygenase.

Materials and Methods

Materials

Zinc deuteroporphyrin 2,4-bis ethylene glycol (ZnDPBG) and chromium mesoporphyrin
(CrMP) were purchased from Frontier Scientific (Logan, UT); these metalloporphyrins were
used for this study as they are potent (51) and selective (3) inhibitors of heme oxygenase activity.
While solubility and photostability (51) make CrMP a preferred choice for in vitro studies,
ZnDPBG is ideal for in vivo experiments because of its quick distribution (52). Isoflurane
(IsoFlo) was obtained from Abbott Laboratories (North Chicago, IL). Thiobutabarbital sodium
(Inactin), and acetylcholine were obtained from Sigma Aldrich (St. Louis, MO). All other
chemicals were obtained from Fisher Scientific (Houston, TX). Acetylcholine (10mmol/l) stock
solution was prepared in saline and diluted in modified Krebs buffer immediately before use. Inactin was prepared fresh daily in saline (50mg/ml). CrMP stock solution (15mmol/l) was prepared in 50mmol/l Na₂CO₃ solution and diluted in modified Krebs buffer (15µmol/l) immediately before use. ZnDPBG solution (15mmol/l) was prepared in 50mmol/l Na₂CO₃ solution for in vivo administration. The composition of modified Krebs buffer was (mmol/l): NaCl 118.5; KCl 4.7; CaCl₂ 1.4; KH₂PO₄ 1.2; MgSO₄ 1.1; NaHCO₃ 25.0; and dextrose 11.1.

Animals

Male lean (Fa/Fa or Fa/fa, n=48) and obese (fa/fa, n=61) Zucker rats (ZR) (Harlan, Indianapolis, IN) were used for the experiments at 13 to 14 weeks of age. Animals were housed in a controlled environment and had free access to standard rodent diet (Harlan Teklad, Madison, WI) and tap water until the day before the experiments. All procedures were approved by the institutional animal care and use committee.

Blood Pressure Measurements in Awake Animals

Male lean and obese ZR were anesthetized with isoflurane and fitted with chronic indwelling femoral arterial catheters (PE-50 tubing filled with heparinized isotonic saline solution) as previously detailed (28). Catheters were tunneled subcutaneously to the nape of the neck and exited via an 18G needle wound and sealed with a steel pin until use. After a three-day surgical recovery period, inline blood pressure was measured daily using a pressure transducer (TSD 104A, Biopac Systems, Santa Barbara, CA) coupled to a polygraph system (Biopac Systems) and a personal computer. Animals were then treated with the heme oxygenase inhibitor ZnDPBG (25µmol/kg/day ip.) or matched vehicle (2ml/kg/day 50mmol/l Na₂CO₃ ip.) for three
days. This particular dose of ZnDPBG was chosen to decrease, but not maximally inhibit heme oxygenase-derived carbon monoxide formation (52).

**Determination of Whole Animal Carbon Monoxide Excretion**

Another group of non-surgerized age matched awake lean and obese ZR were treated with the heme oxygenase inhibitor ZnDPBG (25µmol/kg/day ip.) or matched vehicle (2ml/kg/day 50mmol/l Na₂CO₃ ip.) for three days for daily measurement of carbon monoxide excretion. Animals were placed in an acrylic airtight chamber with the outflow leading to a heated mercuric oxide bed coupled with a gas chromatograph (Peak, Mtn. View, CA) for the determination of carbon monoxide concentration, as has been detailed elsewhere (54,55). The chamber was continuously purged with purified air and the outflow sampled for carbon monoxide concentration at two minute intervals. After 10 minutes of equilibration, the average of four measurements were used to calculate the carbon monoxide excretion rate for the whole animal.

**Metabolic Parameters Measurements and Tissue Extractions**

On the day of the experiment, rats were weighed and anesthetized with thiobutabarbital (Inactin, 120mg/kg ip.) and a carotid arterial catheter was implanted for blood sample collections. Blood samples were drawn for immediate determination of non-fasting blood glucose (Accu-Chek Compact, Roche Diagnostics, Indianapolis, IN), glycated hemoglobin (HbA1c, DCA 2000+ analyzer, Bayer, Pittsburg, PA), and carboxyhemoglobin (HbCO) levels (OSM3 analyzer, Radiometer America, Inc., Westlake, OH), and lipid profile (Cardio Chek PA analyzer, QAS, Orlando, FL). Additional blood was collected in test tubes containing EDTA and plasma was collected by centrifugation. Plasma samples were aliquoted and stored at -20°C until
analyzed. Plasma insulin (rat insulin EIA kit, Cayman Chemical, Ann Arbor, MI) and oxidized LDL (competitive oxidized LDL ELISA kit, Mercodia Inc, Winston Salem, NC) were later determined. Animals were then heparinized (1000U/kg iv.), and the heart, right kidney, thoracic aorta and the gracilis anticus muscles were removed and placed into ice-cold modified Krebs buffer. Right kidney and heart wet weights were then determined.

**Heme Oxygenase-1 ELISA**

Thoracic aorta segments were removed as described above, snap frozen in liquid N\(_2\) and stored at -70°C until analyzed. Aorta samples were powderized in liquid N\(_2\) using a mortar and a pestle. Heme oxygenase-1 protein concentration was determined using the Stressgen rat heme oxygenase-1 ELISA kit (Stressgen, San Diego, CA) and protein concentration was determined using a micro BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Heme oxygenase-1 content was expressed as ng of heme oxygenase-1 protein per mg of total protein.

**Isolated Microvessel Experiments**

Segments of first-order gracilis muscle arterioles were isolated by microdissection (45) and cannulated at both ends with glass micropipets in a vessel chamber (Living Systems Instrumentation, Burlington, VT). The vessel chamber was continuously superfused with gassed buffer (14% O\(_2\) – 5% CO\(_2\) – balanced with N\(_2\); 37°C) via a nonrecirculating system. For internal diameter measurements, the vessel chamber was mounted on a stage of an inverted microscope (Nikon TS 100-F) fitted with a CCD video camera. The camera was connected to a personal computer equipped with video dimensioning software (ImagePro Express, Media Cybernetics). With this setup a magnified image of the arteriolar segment was performed on the computer monitor and internal diameter was measured by manually adjusting guides superimposed by the
video dimensioning software. The software collected images at 1 frame/sec that were stored as digital video files for documentation.

For experiments with no luminal flow, the proximal micropipet was connected to a pressure servo controller (Living Systems Instrumentation) and the distal micropipet to a closed stopcock to achieve and maintain 80 mmHg constant luminal pressure with no flow. After a 60-minute stabilization period, the heme oxygenase inhibitor, 15 µmol/l CrMP, or vehicle was included in the superfusion buffer 20 min before the experiment. This treatment regime was continued throughout the experiment. After the pretreatment period, increasing concentrations of the endothelium-dependent vasodilator, acetylcholine (1 nmol/l – 3 µmol/l) were added to the superfusion buffer. Each concentration was tested for 5 min, internal diameter was recorded every minute and the average of the last two measurements was used to determine the response.

To study flow-induced dilation, both the proximal and distal micropipets were connected to pressure servo controllers and an inline micro flowmeter (Living Systems Instrumentation). The heme oxygenase inhibitor 15 µmol/l CrMP alone, the heme oxygenase inhibitor and the heme oxygenase product carbon monoxide (mean system concentration 100 µmol/l), or vehicle was included in the luminal perfusion buffer. During a 60-minute stabilization period, both proximal and distal pressures were adjusted to 80 mmHg with no luminal flow. During the experiments, proximal and distal pressures were adjusted equally in opposite direction to maintain the midline pressure at 80 mmHg and establish graded levels of luminal flow (0 – 50 µl/min in 5 µl/min increments). Each flow was tested for 5 min, internal diameter was recorded every minute and the average of the last two measurements was used to determine the response. Carbon monoxide concentration was determined at the inflow and outflow ports of the
microvessel micropipets and mean concentration was calculated for the system. In these flow experiments, drugs were only delivered via the luminal perfusion. Carbon monoxide concentration decreased from 200µmol/l to 0µmol/l while passing through the microvessel most likely due to diffusion through the thin arteriolar wall.

Statistics

All data are expressed as mean±SEM. Blood pressure and carbon monoxide excretion measurements, and vascular responses were analyzed by ANOVA using a computer statistical package (Sigma Stat 3.0). When significant differences were observed, orthogonal contrasts were performed as a post hoc analysis (44). All other data were analyzed by t tests, or when equal variance or normality tests failed by Mann-Whitney Rank Sum tests. A value of P<0.05 was considered statistically significant.

Results

Blood Pressure and Carbon Monoxide Excretion Measurements

Awake obese ZR had higher blood pressures (P < 0.05), respiratory carbon monoxide excretion rates (P < 0.05) and HbCO levels compared to lean ZR. In obese ZR rats administration of the heme oxygenase inhibitor ZnDPBG (25µmol/kg/day ip.) lowered mean arterial pressure without affecting heart rate (Figure 1. panel A). In obese ZR the heme oxygenase inhibitor ZnDPBG (25µmol/kg/day ip.) also lowered carbon monoxide excretion rates (Figure 1. panel A). In age matched lean ZR rats the heme oxygenase inhibitor did not affect blood pressure (Figure 1. panel B), or carbon monoxide excretion rates (Figure 1. panel B).
Body and Organ Weight Measurements

Table 1 summarizes the body, kidney and heart weights of lean and obese ZR after 3 days of treatment with the heme oxygenase inhibitor, ZnDPBG (25µmol/kg/day ip.) or matched vehicle. Obese ZR had higher body weights, as well as kidney and heart weights compared to lean ZR. ZnDPBG treatment did not affect body or organ weights in either group.

Metabolic Parameters Measurements

Table 2 summarizes metabolic parameters in lean and obese ZR after 3 days of treatment with the heme oxygenase inhibitor, ZnDPBG (25µmol/kg/day ip.) or matched vehicle. Non-fasting blood glucose, HbA1c, as well as insulin levels were higher in obese ZR compared to lean ZR, and ZnDBPG treatment did not affect these levels in either group. Total cholesterol and LDL cholesterol, but not HDL cholesterol levels were also higher in obese ZR. ZnDPBG treatment did not affect total or HDL cholesterol levels in either group. However, ZnDPBG treatment lowered LDL cholesterol levels in obese, but not in lean ZR. Oxidized LDL levels were also higher in obese ZR, and ZnDPBG did not affect these levels in either group. Triglyceride levels were also elevated in obese ZR and exceeded the upper measurement limit of the Cardio Check PA meter in every case. ZnDPBG treatment did not significantly affect triglyceride levels in either group.

Heme oxygenase-1 ELISA
Figure 2 shows heme oxygenase-1 protein levels in thoracic aortas isolated from lean and obese ZR after 3 days of treatment with the heme oxygenase inhibitor, ZnDPBG (25µmol/kg/day ip.) or matched vehicle. Vascular heme oxygenase-1 protein levels were not significantly different between lean and obese ZR. ZnDPBG treatment promoted an approximately two-fold increase in heme oxygenase-1 protein levels in both groups.

**Isolated Microvessel Experiments**

First-order gracilis muscle arterioles isolated from obese ZR had larger passive internal diameters (lean: 198±4µm, n=14 versus obese: 210±3µm, n=21). During the 60-minute equilibration period diameters of both lean and obese ZR arterioles decreased spontaneously. While the active internal diameters were larger in obese ZR arterioles (lean: 142±7µm versus obese: 159±4µm), there was no difference in myogenic tone between the two strains (lean: Δ\text{max} -62±7µm versus obese: Δ\text{max} -56±4µm). Twenty-minute pretreatment with the heme oxygenase inhibitor, 15µmol/l CrMP, promoted similar vasoconstriction in both groups (lean: Δ\text{max} -37±18µm versus obese: Δ\text{max} -40±4µm).

An endothelium-dependent vasodilator, acetylcholine (1nmol/l – 3µmol/l) promoted concentration-dependent vasodilation in arterioles isolated from lean and obese ZR (Figure 3 panel A). However, acetylcholine-induced vasodilation was greatly attenuated in obese ZR arterioles compared to the lean group (lean: Δ\text{max} 62±7µm; n=5 versus obese: Δ\text{max} 32±4µm; n=8; P < 0.05). Acute in vitro pretreatment with an inhibitor of heme oxygenase, 15µmol/l CrMP, enhanced maximal responses in obese ZR arterioles and abolished the difference between lean
and obese groups (lean: $\Delta_{\text{max}}$ 58±13µm; n=5 versus obese: $\Delta_{\text{max}}$ 59±8µm; n=7) (Figure 3 panel B).

Increases in luminal flow (0 – 50µl/min) promoted vasodilation in arterioles isolated from lean ZR, but not in arterioles from obese animals (lean: $\Delta_{\text{max}}$ 22±2µm; n=4 versus obese: $\Delta_{\text{max}}$ -3±2µm; n=6; P < 0.05) (Figure 4 panel A). Acute in vitro pretreatment with an inhibitor of heme oxygenase, 15µmol/l CrMP, restored flow-induced responses in obese ZR arterioles and abolished the difference between lean and obese groups (lean: $\Delta_{\text{max}}$ 20±2µm; n=5 versus obese: $\Delta_{\text{max}}$ 21±2µm; n=6) (Figure 4 panel B). However, acute in vitro simultaneous pretreatment with the heme oxygenase inhibitor, 15µmol/l CrMP, and the heme oxygenase product, 100µmol/l carbon monoxide, prevented the restoration of flow-induced dilation in obese ZR arterioles ($\Delta_{\text{max}}$ -1±3µm; n=4) (Figure 4 panel B).

**Discussion**

This study is the first to document that endogenous carbon monoxide production is increased in obese ZR with metabolic syndrome, and administration of a heme oxygenase inhibitor lowers respiratory carbon monoxide excretion, as well as blood pressure in awake, obese ZR. Endothelium-dependent vasodilator responses are decreased in skeletal muscle arterioles isolated from obese ZR. Furthermore, acute *in vitro* treatment with a heme oxygenase inhibitor restores endothelial function of resistance vessels to lean ZR levels, but exogenous carbon monoxide prevents these effects. These findings suggest that heme-derived carbon monoxide formation is increased in obese ZR with metabolic syndrome and contributes to hypertension and endothelial dysfunction of resistance vessels. Previous studies suggested that
formation of heme oxygenase-derived carbon monoxide, an established inhibitor of endothelial nitric oxide production, is increased during salt-sensitive forms of hypertension and contributes to arteriolar endothelial dysfunction and hypertension (21,22,48). The current study extends those findings into an experimental model of metabolic syndrome.

Due to a non-functional leptin receptor gene (19), the obese ZR is a well established genetic model of obesity (58) and metabolic syndrome (29,13). Consistent with this, we found that obese ZR exhibit all elements of metabolic syndrome, such as increased body weight, elevated blood glucose, insulin, triglycerides, total, as well as LDL cholesterol levels, and hypertension. Metabolic syndrome represents a major risk factor for the development of type 2 diabetes. A previous study reported that patients with type 2 diabetes show increased respiratory carbon monoxide levels (39). Although the blood glucose levels in these obese ZR are not as high as in other experimental diabetes models, we did find that respiratory carbon monoxide excretion, as well as HbCO levels, is increased in obese ZR indicative of increased endogenous carbon monoxide formation.

The primary endogenous source of carbon monoxide formation is the enzymatic breakdown of heme by heme oxygenase (47). Our previous studies suggested that increased heme oxygenase-derived carbon monoxide formation contributes to salt-induced hypertension in Dahl salt-sensitive rats (21,48). This prompted us to examine the contribution of heme-derived carbon monoxide formation to hypertension in obese ZR. We chose the heme oxygenase inhibitor, ZnDPBG because of its quick tissue distribution (52) and low toxicity. In Sprague-Dawley rats systemic administration of larger doses (45µmol/kg/12hr ip) of ZnDPBG increased blood pressure (28) due to decreased central nervous system carbon monoxide formation and the
subsequent increase in sympathetic outflow (25). In the current study we chose a lower dose of ZnDPBG (25µmol/kg/24hr ip), which decreases respiratory carbon monoxide excretion (52), but minimizes central nervous system effects (52). We found that administration of a heme oxygenase inhibitor, ZnDPBG, reduced respiratory carbon monoxide excretion and lowered blood pressure in awake, obese ZR. These data suggest that increased heme oxygenase-derived carbon monoxide production might contribute to hypertension in obese ZR.

A potential mechanism by which the heme oxygenase inhibitor could lower the blood pressure in obese ZR is through the improvement of metabolic parameters. Although ZnDPBG treatment did lower LDL cholesterol levels in obese ZR, body weight, as well as the rest of the other metabolic parameters (blood glucose, HbA1c, plasma insulin, total cholesterol, HDL cholesterol, and triglycerides levels) were not significantly affected by the heme oxygenase inhibitor. These data argue against major metabolic mechanisms for the blood pressure lowering effect of heme oxygenase inhibition.

Our previous studies in Dahl salt-sensitive rats suggested that increased heme-derived carbon monoxide production contributes to salt-induced hypertension (48) by promoting endothelial dysfunction in resistance vessels (21,48). Decreased endothelium-derived nitric oxide-mediated vasodilation in skeletal muscle arterioles has been suggested to contribute to hypertension in obese ZR (12-14). Thus, we decided to examine if heme oxygenase-derived carbon monoxide contributes to arteriolar endothelial dysfunction in obese ZR. We similarly found that responses to an endothelium-dependent vasodilator, acetylcholine, were attenuated in first-order gracilis muscle arterioles isolated from obese ZR. While acetylcholine is widely used to assess nitric oxide-mediated endothelial function, previous studies suggested that pathological
states can impair muscarinic receptor signaling proximal to nitric oxide synthase (46). An alternative method to circumvent this potential feature is to generate nitric oxide by increasing shear forces along the vascular endothelium. We found that skeletal muscle arterioles from obese ZR failed to dilate in response to flow. Since both acetylcholine and flow-induced (26) dilation can be abolished by nitric oxide synthase inhibition, these data suggest that obese ZR show impaired endothelium-dependent nitric oxide-mediated vasodilation.

To test whether heme oxygenase contributes to endothelial dysfunction in obese ZR we employed another inhibitor, CrMP. This metalloporphyrin is a selective (3) and photostable (51) inhibitor of heme oxygenase activity which was reported to decrease carbon monoxide formation in rat first-order gracilis muscle arterioles (57). We found that acute in vitro application of this heme oxygenase inhibitor restored acetylcholine and flow-induced vasodilator responses in obese ZR and abolished the differences between lean and obese ZR arterioles. These data suggest that a vascular heme oxygenase product contributes to endothelial dysfunction in obese ZR.

Recent studies indicate the physiologically relevant concentrations of carbon monoxide (0.5-50µmol/l) (54,55) can inhibit nitric oxide synthase (37,56), promote endothelial nitric oxide synthase-dependent vasoconstriction (23,42) and impair endothelium-dependent vasodilation (49). We have previously reported that heme-derived carbon monoxide impairs flow-induced dilation in normotensive rat arterioles (26). We have now found that CrMP, a heme oxygenase inhibitor, restores flow-induced dilation in arterioles from obese ZR. To test whether heme oxygenase-derived carbon monoxide mediates endothelial dysfunction in obese ZR arterioles we employed simultaneous treatment with the heme oxygenase inhibitor and exogenous carbon
monoxide. Carbon monoxide prevented the restoration of endothelial function by the heme oxygenase inhibitor, suggesting that heme oxygenase-dependent endothelial dysfunction in obese ZR arterioles is most likely mediated by carbon monoxide.

The two major isoforms of heme oxygenase are the inducible heme oxygenase-1 and the constitutive heme oxygenase-2 (35). Pathological conditions (35), such as oxidative stress (2), diabetes (17) and salt-sensitive hypertension (21,22,48), induce heme oxygenase-1 expression, which can lead to enhanced carbon monoxide formation (38). However, heme oxygenase-derived carbon monoxide formation is normally limited by the availability of heme substrate (27), and heme oxygenase activity can increase in the absence of enhanced heme oxygenase-1 expression (38,54). In the current study, we did not find differences in thoracic aorta heme oxygenase-1 protein levels between lean and obese ZR. However, the lack of an increase in total vascular heme oxygenase-1 protein content does not detract from pathophysiological significance of the elevated \textit{in vivo} production of carbon monoxide.

The effects of increased heme oxygenase-1 activity may not be exclusively adverse as numerous studies have emphasized the cardiovascular protective effects of heme oxygenase-1 induction (10). Certainly heme oxygenase-1 derived carbon monoxide can exert antiproliferative effects in vascular smooth muscle cells and it serves an important protective role against neointima formation following balloon injury or atherosclerosis (9). Other studies have demonstrated the direct vasodilator effects of carbon monoxide on vascular smooth muscle (15). In fact, we were the first to demonstrate the major contributions of endogenous carbon monoxide formation to maintenance of basal resistance vascular diameter (32). It is important to clarify that this vasodilator component of carbon monoxide is maximal in the absence of a functional nitric
oxide system after mechanical denudation of the endothelium (balloon injury, atherosclerosis, or experimental removal) or inhibition of nitric oxide synthesis like in our initial studies (32). Our current study does not address the potential benefits of increased heme oxygenase activity in the obese ZR, but rather clarifies that such benefits may be at least partially undermined by its tendency to promote endothelial dysfunction and hypertension.

We have avoided some common manipulations of the heme oxygenase system. Some studies have used a commercially available heme preparation (heme-L-arginate) to induce heme oxygenase-1 and increase heme oxygenase activity (30). While heme-L-arginate does increase heme-derived carbon monoxide formation, it also contains an abundance of the nitric oxide synthase substrate, L-arginine (1:8 = heme:L-arginine ratio). Since heme is not covalently bound to the L-arginine, and because L-arginine and carbon monoxide compete for binding to nitric oxide synthase (37), the excess L-arginine confers protection against the inhibitory effects of carbon monoxide on nitric oxide synthase (23). Another increasingly popular experimental approach is to induce heme oxygenase-1 expression using various metalloporphyrins. For example, a recent paper has shown the protective effects of cobalt protoporphyrin (CoPP) treatment on endothelial function in a model of type 1 diabetes (50). While CoPP, just as many other metalloporphyrins (35), does increase heme oxygenase-1 protein levels, it is also a potent inhibitor of heme oxygenase activity (36). In fact, our preliminary studies show that, despite increased heme oxygenase-1 expression, systemic administration of CoPP lowers respiratory carbon monoxide excretion and tissue carbon monoxide content. Thus, the protective effects of CoPP treatment in diabetes may be due to decreased endogenous carbon monoxide production just as in our current study. Our current study shows that although ZnDPBG, just as CoPP and
other metalloporphyrins heme oxygenase inhibitors (35), treatment increases vascular heme oxygenase-1 protein levels in obese ZR, respiratory carbon monoxide excretion is in fact decreased in these animals. This also underscores the importance of using in vivo assessments of enzyme activity, instead of solely relying on heme oxygenase-1 expression as a marker of endogenous carbon monoxide production.

Previous studies suggested two other major mechanisms for decreased endothelium-derived nitric oxide-mediated vasodilation in obese ZR arterioles. Oxidative stress has been documented in obese ZR (13), and it is suggested to promote endothelial dysfunction by directly chelating nitric oxide (13,14). However, oxidative stress is also a potent inducer of heme oxygenase-1 expression (2) and also promotes the formation of oxidized LDL (7), which can promote endothelial dysfunction (7), as well as induce heme oxygenase-1 expression (1). In the current study we found that plasma oxidized LDL levels are elevated in obese ZR. Therefore, the possibility exists that oxidative stress promotes endothelial dysfunction not just by direct chelation of nitric oxide, but also by increasing vascular heme oxygenase-derived carbon monoxide formation. The other major mechanism for decreased endothelium-derived nitric oxide-mediated vasodilation in obese ZR arterioles is mediated by the betaII isoform of protein kinase C (5,6). Recently, protein kinase C has been shown to increase heme oxygenase activity (4,34), as well as heme oxygenase-1 levels (34). Therefore, the possibility exists that part of the effects of protein kinase C on endothelial nitric oxide production are mediated by increased heme oxygenase-derived carbon monoxide production. Thus, our current results are not proposing an alternate mutually exclusive mechanism for endothelial dysfunction in obese ZR, but rather may identify a common mediator downstream from the other previously described mechanisms. This
might also explain why other treatments, such as reducing oxidative stress by superoxide
dismutase, only confer partial restoration of endothelial function (12-14).

In summary, we found that endogenous carbon monoxide production is increased in
obese ZR with metabolic syndrome and that administration of a heme oxygenase inhibitor to
“normalize” carbon monoxide excretion lowers blood pressure in awake, obese ZR. In addition,
we find that endothelium-dependent vasodilatory responses are decreased in skeletal muscle
arterioles isolated from obese ZR. Furthermore, acute in vitro treatment with a heme oxygenase
inhibitor restores endothelium-dependent dilation to lean ZR levels, but exogenous carbon
monoxide prevents these effects. These findings suggest that heme-derived carbon monoxide
formation is increased in obese ZR with metabolic syndrome and contributes to hypertension and
endothelial dysfunction in resistance vessels. Our results may help to identify novel therapeutic
targets to improve endothelial function and treat hypertension in patients with metabolic
syndrome.
Acknowledgments

This work was supported by National Institutes of Health grants: an Institutional Development Award (Idea) Program P20 RR017659 (FK Johnson) from the National Center for Research Resources, and National Heart, Lung and Blood Institute grants R01 HL76187 (RA Johnson), and R01 HL59976 (W Durante) and R01 HL74966 (W Durante), and fellowship F32 HL76001 (KE Jackson); and by the American Heart Association’s Established Investigator Grant (W Durante).
References


12. **Frisbee JC.** Reduced nitric oxide bioavailability contributes to skeletal muscle microvessel rarefaction in the metabolic syndrome. *Am J Physiol Regul Integr Comp Physiol* March 31, 2005; 10.1152/ajpregu.00114.2005


Figure Legends

Figure 1. Heart rate (top panels), mean arterial pressure (middle panels), and carbon monoxide excretion rate (bottom panels) of awake obese ZR (panel A) or lean ZR (panel B) before (Day 0) and after (Days 1-3) intraperitoneal administration of vehicle (open circles, obese n=7 for blood pressure and n=5 for carbon monoxide, lean n=5 for blood pressure and n=4 for carbon monoxide) or an inhibitor of heme oxygenase, 25µmol/kg/day zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, closed circles, obese n=7 for blood pressure and n=5 for carbon monoxide, lean n=4 for both). Data are expressed as mean±S.E.M. * indicates P<0.05 relative to vehicle.

Figure 2. Heme oxygenase-1 protein content in thoracic aortas isolated from lean (left two bars) and obese ZR (right two bars) after 3 days of treatments with vehicle (open bars, lean n=4, obese n=5) or an inhibitor of heme oxygenase, 25µmol/kg/day zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, closed bars, lean n=4, obese n=5). Data are expressed as mean±S.E.M. * indicates P<0.05 relative to vehicle.

Figure 3. Concentration-dependent effects of an endothelium-dependent vasodilator, acetylcholine (ACh), on changes in internal diameter of first-order gracilis muscle arterioles isolated from lean (open circles) or obese (closed circles) Zucker rats. Arterioles were acutely pretreated in vitro with vehicle (panel A, lean n=5, obese n=8), or with an inhibitor of heme oxygenase, 15µmol/l chromium mesoporphyrin (CrMP) (panel B, lean n=5, obese n=7) for 20 minutes prior to ACh administration. Data are expressed as mean±S.E.M. change in diameter. * indicates P <0.05 relative to lean group.
Figure 4. Flow-induced changes in internal diameter of first-order gracilis muscle arterioles isolated from lean (open circles) or obese (closed circles) Zucker rats. Arterioles were acutely pretreated in vitro with vehicle (panel A, lean n=4, obese n=6), with an inhibitor of heme oxygenase, 15µmol/l chromium mesoporphyrin (CrMP) (panel B, lean n=5, obese n=6), or simultaneously with CrMP and a heme oxygenase product, 100µmol/l carbon monoxide, (panel B closed squares, obese n=4), for 20 minutes prior to initiation of flow. Data are expressed as mean±S.E.M. * indicates P<0.05 relative to lean group. † indicates P<0.05 relative to obese CrMP pretreated arterioles.
**Table 1** Body and organ weights of lean and obese Zucker rats after 3 days of vehicle or ZnDPBG treatments.

<table>
<thead>
<tr>
<th></th>
<th>Lean ZR</th>
<th>Obese ZR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (n=4)</td>
<td>ZnDPBG (n=4)</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>294±11</td>
<td>303±6</td>
</tr>
<tr>
<td>Kidney Weight (g)</td>
<td>0.99±0.07</td>
<td>0.98±0.02</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>0.90±0.03</td>
<td>0.91±0.02</td>
</tr>
</tbody>
</table>

* indicates P<0.05 obese vehicle compared to lean vehicle group
Table 2 Metabolic parameters of lean and obese Zucker rats after 3 days of vehicle or ZnDPBG treatments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lean ZR Vehicle (n=4)</th>
<th>Lean ZR ZnDPBG (n=4)</th>
<th>Obese ZR Vehicle (n=5)</th>
<th>Obese ZR ZnDPBG (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>159±4</td>
<td>151±11</td>
<td>178±6*</td>
<td>176±15</td>
</tr>
<tr>
<td>HbA1c (%)§</td>
<td>3.3±0.0</td>
<td>3.3±0.1</td>
<td>4.1±0.4*</td>
<td>4.0±0.1</td>
</tr>
<tr>
<td>HbCO (%)</td>
<td>3.0±0.1</td>
<td>-</td>
<td>3.9±0.1*</td>
<td>-</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>68±3</td>
<td>65±3</td>
<td>97±7*</td>
<td>100±1</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dl)</td>
<td>20±2</td>
<td>18±3</td>
<td>35±9</td>
<td>50±2</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dl)</td>
<td>48±1</td>
<td>46±1</td>
<td>61±3*</td>
<td>50±2 †</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>27±2</td>
<td>61±26</td>
<td>500±0*‡</td>
<td>362±91</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>2.7±0.8</td>
<td>1.5±0.1</td>
<td>56.7±13.9*</td>
<td>21.3±13.0</td>
</tr>
<tr>
<td>Oxidized LDL (U/l)</td>
<td>3.5±0.4</td>
<td>3.6±0.1</td>
<td>8.1±0.3*</td>
<td>8.5±0.4</td>
</tr>
</tbody>
</table>
HbA1c = glycated hemoglobin, HbCO = carboxyhemoglobin, HDL = high density lipoprotein, LDL = low density lipoprotein

* indicates \( P < 0.05 \) obese vehicle compared to lean vehicle group
† indicates \( P < 0.05 \) ZnDPBG compared to corresponding vehicle treated group
‡ upper limit of triglyceride measurements is 500mg/dl
§ separate set of animals, lean vehicle \( n = 6 \), lean ZnDPBG \( n = 7 \), obese vehicle \( n = 5 \), obese ZnDPBG \( n = 5 \)
Figure 1.
Figure 2.
Figure 3.
Figure 4.