Hypertension is an important prevalence risk factor for many cardiovascular diseases, including myocardial infarction, heart failure, and coronary artery diseases. Effective control of established hypertension has not been an easy target. Existing antihypertensive therapies require hypertensive patients to go through lifelong treatment. Discontinuation of antihypertensive therapies usually leads to a quick return of hypertension. On the other hand, sustained application of antihypertensive therapies often results in severe adverse effects and high financial costs, which underlines the noncompliance of hypertension patients to antihypertensive therapies. This dilemma emphasizes the importance of searching for a long-lasting antihypertensive therapy.

Endogenous carbon monoxide (CO) production is regulated by heme oxygenase (HO). Heme or its Fe(III) oxidation product hemin is catalytically broken down by HO to yield CO, bilirubin, and iron. HO has 3 distinct isoforms, namely, inducible HO-1 and constitutive HO-2 and HO-3. The 32-kDa HO-1 protein plays a predominant role for CO generation under pathophysiological conditions. CO has been shown to induce relaxation of vascular smooth muscle cells by stimulating soluble guanylyl cyclase (sGC) or opening calcium-activated K⁺ channels. Being potent antioxidants, bilirubin and biliverdin may reduce hypertension severity by lowering oxidative stress, inhibiting lipid peroxidation, and increasing the half-life of NO.

Previous reports showed that the administration of HO inducers like hemin or SnCl₂ for 4 consecutive days restored blood pressure (BP) to the physiological level in young (8 weeks) but not in adult spontaneously hypertensive rats (SHRs). The mechanisms for this age-dependent effectiveness of HO-1 inducers on the regulation of BP remain unclear. Because transcription and translation of some proteins may be slower in adults, the activity/expression of these proteins unaffected by a given acute treatment may be increased by the same treatment but with longer duration. Our recent study showed that prolonging the hemin administration period of 13 days lowered, albeit not yet normalized, high BP of 12-week-old SHR. It was perceived that an even longer hemin treatment period might normalize BP and may have a long-lasting effect in SHR with established hypertension.

In this study, we report on normalization of high BP in adult SHRs that occurred during 21 days of continuous hemin administration, namely, the hemin protocol, and lasted for 9 months. Multiple underlying mechanisms were investigated to determine the long-lasting antihypertension effect of the hemin protocol.

Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000239673.80332.2f
Animal Preparation
Male SHRs of 12-week-old (n=80) and age-matched Sprague-Dawley (SD) rats (n=70; Charles River Laboratories, Willington, MA) were used in this study. These animals were housed at 21°C with 12-hour light/dark cycles, fed with standard laboratory chow, and had access to drinking water ad libitum.

Hemin solution was prepared as reported previously. Hemin was administered (15 mg/kg per day) through subcutaneously implanted osmotic minipumps (Model 2ML4, Alzet) for 21 consecutive days to administer (15 mg/kg per day) through subcutaneously implanted osmotic minipumps (Model 2ML4, Alzet) for 21 consecutive days to 12-week-old SHRs (n=20) and SD rats (n=20). In another experiment, hydralazine (45 mg/kg per day) was given orally for 21 days to 12-week-old SHRs (n=20) and age-matched SD rats (n=10). Sham control groups, composed of 12-week-old SHR and SD rats, were administered with vehicle solution of 0.9% NaCl (2 mL) using implanted osmotic minipumps (n=20 per group) for 21 consecutive days. The rest of animals (20 SHRs and 20 SD rats) were left untreated as age control.

BP Measurement
Systolic BP was measured in conscious rats using a standard tail-cuff noninvasive BP measurement system (Model 29-SSP, Harvard Apparatus) after acclimatization, 2 days before the start of the hemin protocol, and successively on a daily basis for the entire 21-day period. At the end of the 21-day period, 10 animals of each group were weighed, anesthetized with pentobarbital sodium (50 mg/kg body weight, IP), and euthanized. The mesenteric arteries adherent to the intestine were isolated. The main branch of the mesenteric tree (superior mesenteric artery) was isolated and cleaned out of the fat tissue and peritoneal coverings. The second branch of the mesenteric tree was then identified down to its terminal branches and then isolated. Thereafter, the isolated and cleaned mesenteric arteries were snap-frozen in liquid nitrogen. The rest of the animals (n=10 per group) were kept for continuous BP monitoring. Subsequent BP measurements were carried out for the remaining observation period. The animal experimental protocol was approved by University of Saskatchewan Standing Committee on Animal Care and Supply.

General Methodologies
Serum heme level was measured by the pyridine-hemochromogen method as described previously. The same protocols for vascular tissue preparation and Western blotting were used as described previously. The concentration of cGMP was determined by a radioimmunoassay kit (125I-cGMP-RIA, Amersham International plc) as described previously.

Serum total bilirubin, alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γGT), urea, and creatinine levels were analyzed within 24 hours by the Laboratory of Clinical Chemistry at Royal Hospital (Saskatoon, Saskatchewan, Canada).

Morphometric Analysis
Rats were anesthetized and the mesenteric arteries dissected and immediately fixed by immersion in 4% paraformaldehyde for 16 to 18 hours. Samples were then incubated in a 30% sucrose solution for 3 days at 4°C. After embedding in OCT compound (Somagen Diagnostics), sections of 8-μm thickness were cut on a cryostat and picked up on poly-L-lysine–coated slides. The circumferences of the vessels were measured after obtaining amplified (×100) images of the sections by a microscope (Olympus 1×70).

Chemicals and Statistical Analysis
Unless specified otherwise, all of the chemicals were purchased from Sigma. All of the data were expressed as mean ± SEM from ≥3 independent experiments performed in duplicate unless otherwise stated. Statistical analyses were done using unpaired Student’s t test, ANOVA in conjunction with Newman–Keuls test for repeated measures where appropriate. Group differences at the level of P<0.05 were considered statistically significant.

Results
Normalization of BP in Adult SHRs During and After the Hemin Protocol
Systolic BP of adult SHRs was lowered to the normotensive level 2 weeks after the start of the hemin protocol (203±3 versus 123±2 mm Hg; n=20). However, the hemin protocol had no effect on age-matched normotensive SD rats (121±2 versus 118±1 mm Hg; n=20; Figure 1A). On the other hand, systolic BP of vehicle-treated SHRs remained in the hypertensive range at the end of the 3-week hemin protocol (212±3 versus 204±2 mm Hg; n=20). Figure 1B shows day-to-day change in systolic BP of hemin-treated SHRs throughout the hemin protocol duration.

The antihypertensive effect of the hemin protocol was sustained after the removal of hemin pumps for 9 months (140±3 mm Hg; Figure 1C). By month 10 after the hemin protocol, systolic BP of SHRs reached 148±2 mm Hg, whereas that of untreated SHRs was 229±2 mm Hg (P<0.001; Figure 1C).

Changes in Serum Heme Levels, Expression of HO Proteins, and HO Activity During and After the Hemin Protocol
Immediately after the start of the hemin protocol, serum heme levels of SHR (n=20) and SD rats (n=20) increased significantly from a nearly undetectable baseline to 4.8±0.1 and 5.1±0.1 μmol/L, respectively (Figure 1D). Two weeks after the removal of hemin pumps, serum heme levels returned gradually to the baseline. Serum heme levels of saline-treated or untreated SHRs and SD rats (n=10 per group) did not change significantly during and after the 3-week treatment/observation period (Figure 1D).

Basal expression level of HO-1 protein in mesenteric arteries was significantly higher in adult SHRs than that in age-matched SD rats. At the end of the 3-week hemin protocol, HO-1 expression was significantly upregulated in the mesenteric arteries from both SHRs and SD rats, with the former being more significant (P<0.01; Figure 2A). In contrast, no difference was detected in the expression levels of the constitutive HO-2 proteins in the mesenteric artery of SHRs and SD rats with or without heme treatment (Figure 2B). Consistent with HO-1 expression change, 3-week hemin protocol significantly increased total HO activity in the mesenteric artery of adult SHRs (Figure 2C). Although an increase in HO activity was also detected in heme-treated SD rats, the amount of increment in the heme-treated SHRs was significantly greater (54% in SD rats versus 300% in SHR; P<0.05).

For the purpose of comparison, adult SHRs were also treated for 3 weeks with hydralazine. Hydralazine normalized systolic BP of adult SHRs 3 weeks after continuous oral administration (184±2 versus 126±2 mm Hg; P<0.01; n=20; Figure 3A). The antihypertensive effect of hydralazine, however, only lasted 2 weeks after the termination of hydralazine administration (135±3 mm Hg; Figure 3A). Thereafter, systolic BP of these SHRs gradually climbed up. At week 4 after hydralazine treatment, systolic BP of hydralazine-treated SHRs returned to the pretreatment level (184±2 versus 179±3 mm Hg; P>0.05; n=10).
The acute antihypertensive effect of hydralazine was not related to the function of the HO/CO system, because at the end of 3-week hydralazine treatment, the expression of HO-1 did not change significantly in the mesenteric arteries in comparison with that of the nontreated age-matched SHRs (Figure 3B). Hydralazine treatment also did not alter the expression levels of HO-2 in the mesenteric arteries of adult SHRs (Figure 3C). It should be noticed that, however, whether or not hydralazine treatment changed total HO activity has not been measured, which may limit the interpretation of our results.

Hemin-induced upregulation of HO-1 in SHRs remained for 9 months after the removal of hemin pumps (*P*<0.05 versus control SHRs; Figure 4A). Similarly, total HO activity remained significantly higher in 55-week-old hemin-treated SHRs than in age-matched untreated SHRs (Figure 4B). Changes in sGC Expression, cGMP Content, and Phosphodiesterase-5 Expression During and After the Hemin Protocol

Adult SHRs showed comparable levels of cGMP content and sGC proteins in vascular tissues with that of age-matched SD rats (Figure 5A and 5B). Expression level of sGC proteins and content of cGMP in vascular tissues of SHRs but not that of hemin-treated SD rats were significantly increased at the end of the 3-week hemin protocol (Figure 5A and 5B). Adult SHRs had a significantly higher phosphodiesterase (PDE)-5 protein expression level in vascular tissues than that in SD rats. The hemin protocol significantly downregulated the expression of PDE-5 in vascular tissue of SHRs but not that of hemin-treated SD rats at the end of the 3-week treatment period (Figure 5C).

Nine months after the removal of hemin pumps, sGC protein expression level and the content of cGMP in the mesenteric artery of adult SHRs remained significantly higher than that of age-matched control SHRs or hemin-treated SD rats (*P*<0.05; Figure 6A and 6B). Downregulated expression of PDE-5 by the hemin protocol in the mesenteric artery of SHR also remained for 9 months after hemin pump removal (Figure 6C).

Changes in Expression Level of Inducible NO Synthase and Endothelial NO Synthase Proteins

Basal expression level of inducible NO synthase (iNOS) proteins in the mesenteric artery of adult SHRs was significantly higher than that of age-matched SD rats (Figure 7A). In contrast, no difference was detected in basal expression of endothelial NO synthase (eNOS) proteins between the mesenteric artery of adult SHRs and SD rats (Figure 7B). At the end of the 3-week hemin protocol, there was no difference in the expression levels of iNOS or eNOS proteins between treated or nontreated age-matched SHRs (Figure 7).

Vascular Remodeling in SHRs

Significant eutrophic inward remodeling of small peripheral mesenteric arteries of adult SHRs was confirmed with decreased lumen diameter and increased wall/lumen ratio in comparison with age-matched SD rats. The hemin protocol resulted in significant reversal of eutrophic inward remodeling of the mesenteric arteries of SHRs at the end of the 3-week treatment. Arterial lumen sizes, wall media thickness, and wall/lumen ratio of hemin-treated SHRs were reversed to the level of that from age-matched normotensive SD rats (Table). The reversed eutrophic inward remodeling of small
Changes in the Expression of Vascular Endothelial Growth Factor-α

Basal expression level of vascular endothelial growth factor (VEGF)-α proteins in vascular tissues of SHRs was higher than...
that of age-matched SD rats. Reduced expression of VEGF-α in the mesenteric artery of SHRs after the 3-week hemin protocol was observed (Figure 8A). Nine months after the removal of hemin pumps, expression level of VEGF-α proteins in the mesenteric arteries of SHRs was still significantly lower than that of age-matched untreated SHRs (P<0.05; Figure 8B). Hemin treatment also significantly lowered the expression of VEGF-α proteins in the mesenteric arteries of SD rats at the end of the 3-week hemin protocol, as well as 9 months later in comparison with that of age-matched untreated SD rats (Figure 8).

**Safety of the Hemin Protocol**

The 3-week hemin protocol did not change body weights of the treated SHRs in comparison with that of age-matched untreated SHRs (P>0.05; data not shown). Plasma ALT and γGT are important markers of hepatotoxicity. Comparable plasma levels of ALT were found in 15-week-old untreated SHRs (n=7) and age-matched treated SHRs at the end of the 3-week hemin protocol (n=7; 44.1±2.1 versus 47.9±3.5 IU/L). Plasma γGT level in untreated 15-week-old SHRs (n=7) was 43.4±5.2 IU/L, whereas it was 49.3±3.8 IU/L in age-matched treated SHRs at the end of the 3-week hemin protocol (n=7; P>0.05). Serum total bilirubin levels were within the reference range (0.2 to 1.2 mg/dL) in treated SHRs at the end of the 3-week hemin protocol (n=7; 0.9±0.02 mg/dL) but significantly greater than that in age-matched 15-week-old untreated SHRs (n=7; 0.4±0.03 mg/dL; P<0.05). Furthermore, in untreated 15-week-old SHRs (n=10), body weight/liver weight was 25.2±0.71, whereas in age-matched treated SHRs at the end of the 3-week hemin protocol (n=7), it was 27.3±2.2 (P>0.05). There was also no significant difference in body weight/kidney weight between hepatotoxic SHRs (98.3±2.3) and the age-matched untreated 15-week-old SHRs (96.5±3.5; P>0.005).

Age-matched 15-week-old untreated SHRs (n=7) and the treated SHRs at the end of the 3-week hemin protocol (n=7) had comparable normal levels of serum urea (14.3±2.2 versus 15.7±1.9 mgN/dL) and creatinine (0.9±0.03 versus 1.0±0.06 mg/dL). Normal serum urea and creatinine levels are 10 to 20 mg nitrogen/dL and 0.7 to 1.4 mg/dL, respectively.

**Discussion**

Different pharmacological and genetic approaches have been used to upregulate HO-1 with the ultimate goal of normalization of BP in SHRs. The usage of stannous chloride for 4 days (IP),
a well-known HO inducer, significantly lowered BP of 7-week-old SHRs but not that of 20-week-old SHRs. Treatment with hemin and hemin arginate of 7-week-old SHRs for 4 days (IP) normalized high systolic BP to 130±5 mm Hg. Systolic BP of these rats reached 140 mm Hg 1 week later and gradually increased to the levels of untreated SHRs 1 month later. All of these previous attempts to combat hypertension by upregulating HO only modestly lowered high BP in young SHRs (5 to 8 weeks old) for an apparently short period of time (3 to 4 weeks). Once hypertension is fully established in SHRs (such as in 20-week-old SHRs), upregulating HO expression using a 4-day IP hemin injection failed to lower BP. More importantly, a long-lasting normalization of BP in the absence of antihypertensive interventions has not been realized clinically or experimentally by either upregulating HO expression or other interventions. In responding to these challenges, we report in this study an unprecedented 9-month normalization of BP in adult SHRs after a 3-week hemin protocol.

Our choice of the 3-week hemin protocol was based on our previous findings that a 13-day hemin administration (IP) significantly lowered systolic BP of 12-week-old SHRs. Furthermore, our preliminary observations indicated that a 3-week-old IP administration of hemin into adult SHRs lowered high systolic BP to a normotensive level. It was found that systolic BP of 12-week-old SHRs was normalized 2 weeks after the implantation of subcutaneous hemin-releasing pump, which is 1 week faster than the administration through the intraperitoneal route in adult SHRs. During the third week of the hemin protocol, there was no further change in systolic BP level in treated SHRs. Restoration of systolic BP in adult SHRs with established hypertension to a normotensive level by HO-1 inducers has not been reported to date, although there are some reports on partial lowering of systolic BP, either with acute treatment for several days or chronic treatment for several weeks, and clearly this challenge has been met by our novel hemin protocol.
Vascular HO-1 expression level in the mesenteric arteries was significantly higher in adult SHRs than age-matched SD rats. The elevated HO-1 expression in adult SHRs may represent a compensatory reaction to counter high shear stress on blood vessels with hypertension. Although hemin treatment also increased the levels of HO-1 in SD rats, there was no corresponding increase in sGC or cGMP levels. It seems that the magnitude of hemin-induced HO-1 upregulation did not surpass a threshold level needed to stimulate further downstream regulatory proteins, such as sGC. Consequently, no BP change was observed in hemin-treated SD rats. Nevertheless, we cannot rule out the effects of integrated functioning of other physiological reactions that may be alerted to maintain normal BP levels in otherwise nortensive SD rats. In our previous report, age-matched nortensive Wistar–Kyoto rats were also treated for 3 weeks with hemin therapy, and no BP change was observed (n=10)\(^{15}\).

There are several explanations for hemin protocol–induced long-lasting normalization of BP in adult SHRs. Certainly, the upregulated HO-1 expression during 3-week hemin protocol is sustained even after the removal of the hemin pump. This could be attributed to prolonged induction of HO-1 expression and/or prolonged stability of the existing HO-1 proteins, triggered by and maintained after the 3-week hemin protocol. The excessive and sustained high levels of HO activity led to a sustained decrease in VEGF expression in resistance blood vessels\(^{16,17}\) in adult SHRs 9 months after hemin pump removal.

VEGF-induced endothelium-dependent relaxation of canine coronary arteries has been reported.\(^{18}\) VEGF preferentially dilates arterioles and venules without affecting medium-sized arteries and veins.\(^{19}\) Intracoronary and intravenous infusions of recombinant human VEGF also significantly lowered systolic BP.\(^{20}\) The hypotensive effect of VEGF has been ascribed to the enhancement of eNOS activity and its expression\(^{21}\) or the impaired baroreflex function and reduced VEGF clearance.\(^{22}\) On the contrary, other recent studies claim that VEGF may be involved in the pathogenesis of hypertension. For example, Zhao et al\(^{16}\) demonstrated that VEGF is an essential mediator in angiotensin II–induced vascular inflammation and structural changes through its proinflammatory actions. Suppression of VEGF attenuated vascular inflammation and remodeling in hypertension. A recent clinical study demonstrated a positive correlation between hypertension and higher VEGF levels.\(^{17}\) It seems in our study that low expression of VEGF suppressed eutrophic remodeling of small peripheral arteries in SHRs.\(^{23}\)

Consequently, the lumen diameter of peripheral mesenteric arteries of SHRs was increased and the media/lumen ratio decreased without any change in the colony-stimulating activity. These intriguing structural changes probably underlie the long-lasting normalization of BP observed in this study. The mechanism by which HO-1 expression remains elevated after the

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**Morphological Changes of the Main Mesenteric Arteries of Hemin-Treated and Untreated Rats**

<table>
<thead>
<tr>
<th>Treatment and Animal Species</th>
<th>Lumen Diameter, mm</th>
<th>Colony-Stimulating Activity, mm(^2)</th>
<th>Media, (\mu)m</th>
<th>Media/Lumen, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control SHRs (15 wk)</td>
<td>10</td>
<td>0.53±0.02</td>
<td>0.15±0.03</td>
<td>84.7±2.4</td>
</tr>
<tr>
<td>Hemin-treated SHRs (15 wk)</td>
<td>10</td>
<td>0.68±0.03*</td>
<td>0.18±0.02</td>
<td>82.7±1.8</td>
</tr>
<tr>
<td>SD rats (15 wk)</td>
<td>10</td>
<td>0.74±0.04†</td>
<td>0.18±0.05</td>
<td>79.3±2.2</td>
</tr>
<tr>
<td>Control SHRs (55 wk)</td>
<td>10</td>
<td>0.52±0.05</td>
<td>0.15±0.04</td>
<td>85.7±1.7</td>
</tr>
<tr>
<td>Hemin-treated SHRs (55 wk)</td>
<td>10</td>
<td>0.64±0.03*</td>
<td>0.17±0.02</td>
<td>83.4±3.2</td>
</tr>
<tr>
<td>SD rats (55 wk)</td>
<td>10</td>
<td>0.71±0.03†</td>
<td>0.18±0.03</td>
<td>78.4±2.4</td>
</tr>
</tbody>
</table>

Chronic hemin treatment significantly decreased eutrophic remodeling of the mesenteric artery in adult SHRs. The lumen diameters of hemin-treated animals were similar to those of age-matched nortensive SD rats and significantly different from untreated SHRs.

\*\(p<0.05\) vs untreated control SHRs.

\(\dagger p<0.05\) vs untreated age-matched control SHRs.

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**Figure 8.** Expression of VEGF-\(\alpha\) proteins in the mesenteric arteries of SHRs and SD rats induced by the hemin protocol. A, Representative Western blot (inset) and summary of relative abundant levels of VEGF-\(\alpha\) proteins in 15-week-old SHRs and SD rats at the end of the 3-week hemin protocol. \(p<0.01\) vs all other groups; \#\(p<0.05\) vs untreated SD rats. n=10 for each group. B, Representative Western blot (inset) and summary of relative abundant levels of VEGF-\(\alpha\) proteins in 55-week-old SHRs and SD rats 9 months after the removal of hemin pumps. \(p<0.01\) vs all other groups; \#\(p<0.05\) vs untreated SD rats. n=10 for each group.
hemin protocol is unclear at the moment. Certainly, it is not because of a continuous elevation of the serum heme level as shown in Figure 1D. Furthermore, at the time of animal scarification, either at the end of the 3-week hemin protocol or 9 months after the hemin protocol, no heme accumulation was visualized at the subcutaneous hemin pump implantation sites. We cannot, however, disregard the possibility yet that infused heme may accumulate in the blood vessel wall locally and metabolized there slowly, which may provide long-lasting stimulation of HO-1 upregulation.

Perspectives
The upregulation of HO-1 expression in peripheral vasculature by the hemin protocol reported in this study offers one mechanism for the sustained antihypertension protection in SHR rats. Because the upregulation of HO-1 expression by the hemin protocol is unlikely limited only to vasculatures, expression and activity of HO-1 in the kidney and other vital organs induced by the hemin protocol may also contribute to the antihypertensive effect of this protocol. Comparison of changes in vascular and renal HO expression induced by the hemin protocol, for example, will help identify tissue-specific responsiveness of HO-1 expression to guide the treatment of different types of hypertension. Further investigation of the application and efficacy of the hemin protocol to other types of experimental hypertension models and human hypertension are also merited, which would eventually lead to a new therapeutic approach for the management of established hypertension with high potency and long-lasting protection.

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Disclosures
A relevant patent application, of which R.W. and L.W. are among the applicants, for the use of hemin treatment to lower blood pressure has been filed by the University of Saskatchewan.

References
Sustained Normalization of High Blood Pressure in Spontaneously Hypertensive Rats by Implanted Hemin Pump

Rui Wang, Rany Shamloul, Xiaoxia Wang, Qinghe Meng and Lingyun Wu

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