The hyperbilirubinemic Gunn rat is resistant to the pressor effects of angiotensin II

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Pflueger, Axel, Anthony J. Croatt, Timothy E. Peterson, Leslie A. Smith, Livius V. d’Uscio, Zvonimir S. Katusic, and Karl A. Nath. The hyperbilirubinemic Gunn rat is resistant to the pressor effects of angiotensin II. Am J Physiol Renal Physiol 288: F552–F558, 2005. First published November 9, 2004; doi:10.1152/ajprenal.00278.2004.—ANG II induces vasoconstriction, at least in part, by stimulating NADPH oxidase and generating reactive oxygen species. ANG II also induces heme oxygenase activity, and bilirubin, a product of such activity, possesses antioxidant properties. We hypothesized that bilirubin, because of its antioxidant properties, may reduce the pressor and prooxidant effects of ANG II. Our in vivo studies used the hyperbilirubinemic Gunn rat which is deficient in the enzyme uridine diphosphate glucuronosyltransferase, the latter enabling the excretion of bilirubin into bile. ANG II (0.5 mg·kg−1·day−1) or saline vehicle was administered by osmotic minipump to control and Gunn rats for 4 wk. The rise in systolic blood pressure induced by ANG II, as observed in control rats, was markedly reduced in Gunn rats, the latter ~50% less at 3 and 4 wk after the initiation of ANG II infusion. The chronic administration of ANG II also impaired endothelium-dependent relaxation responses in control rats but not in Gunn rats. As assessed by the tetrahydrobiopterin/dihydrobiopterin ratio, ANG II induced oxidative stress in the aorta in control rats but not in Gunn rats. Heightened generation of superoxide anion in aortic rings in ANG II-infused rats and by vascular smooth muscle cells exposed to ANG II was normalized by bilirubin in vitro. We conclude that the pressor and prooxidant effects of ANG II are attenuated in the hyperbilirubinemic Gunn rat, an effect which, we speculate, may reflect, at least in part, the scavenging of superoxide anion by bilirubin.

NADPH oxidase; heme oxygenase; nitric oxide; endothelial function

ANG II ACTIVATES THE NADPH oxidase enzyme system and promotes the generation of superoxide anion and other reactive oxygen species (15, 25, 36, 40, 43). Such stimulation of superoxide anion contributes significantly to vasoconstrictive and pressor effects of ANG II, as demonstrated by studies that use antioxidants including those that scavenge the superoxide anion (25, 36, 40, 43). Indeed, it is generally accepted that systemic hypertension induced by the chronic administration of ANG II in rodents arises, at least in part, from the generation of superoxide anion as NADPH oxidase is activated by ANG II (25, 36, 40, 43).

Our prior studies as well as the studies of others demonstrate that systemic administration of ANG II leads to marked up-regulation of heme oxygenase-1 (HO-1) (4, 15, 49). HO is the rate-limiting enzyme in the degradation of heme, enabling the conversion of heme to biliverdin, in the course of which carbon monoxide evolves and iron is liberated from the heme ring; subsequently, biliverdin is converted to bilirubin (2, 3, 31, 32, 39). The HO-1 isoform is rapidly induced by diverse stimuli and often confers a cytoprotective, anti-inflammatory, and vasorelaxant effect (2, 3, 31, 32, 39). The induction of HO-1 following the administration of ANG II likely exerts a countervailing, vasorelaxant effect: prior upregulation of HO-1 reduces the rise in systemic blood pressure induced by ANG II, whereas inhibition of HO activity exacerbates the increase in systemic blood pressure induced by ANG II (4, 15, 49).

Although the basis for this attenuating effect of induced HO-1 on ANG II-induced systemic hypertension is uncertain, several possibilities exist (1, 2, 12, 28, 39, 51). Upregulation of HO-1 leads to the generation of carbon monoxide and cyclic GMP which possess potent vasorelaxant effects. Upregulation of HO-1 also inhibits the production of vasoconstrictors such as endothelin-1, thromboxanes, isoprostanes, 19-HETE, 20-HETE, and PDGF. Additionally, HO activity is linked to the Kca channel which mediates vasorelaxant actions.

Another possibility whereby increased HO activity may provide vasorelaxant effects that oppose the vasoconstricting actions of ANG II is through the generation of bile pigments, specifically, bilirubin. Indeed, our prior studies demonstrate that ANG II not only induces HO-1 mRNA and protein but also HO activity, the latter specifically demonstrated by increased generation of bilirubin by tissue microsomes harvested from ANG II-infused rats (15). Dating from the original studies of Stocker et al. (41, 42) some 20 years ago, the oxidant-scavenging properties of bilirubin are well recognized. Such antioxidant effects are demonstrable in studies undertaken in vitro and in vivo: for example, bilirubin reduces oxidative stress in renal proximal tubular epithelial cells, as shown in our prior studies (26), and in other cell types (37, 47). The administration of bilirubin can acutely protect against oxidative stress in organs in vivo (16, 27, 48), and marked elevations in plasma bilirubin achieved by common bile duct ligation, as shown in our prior studies, are associated with protection against heme protein-induced renal injury (26).

In the current study, we hypothesize that bilirubin may mitigate the pressor effects of ANG II by scavenging ANG II-induced generation of reactive oxygen species. In these studies, we used the inbred homozygous Gunn rat, which exhibits marked elevations in plasma bilirubin levels (18, 19). This mutant strain lacks the enzyme uridine diphosphate glu-
curonosyl transferase (UDPGT), which enables the excretion of bilirubin into bile, and thus exhibits marked hyperbilirubinemia due to elevated plasma levels of unconjugated bilirubin. This mutant strain provides an established approach in assessing the mechanisms underlying and pathophysiological effects of hyperbilirubinemia (9, 18, 19, 52), having been employed, for example, in studies that demonstrate the efficacy of bilirubin as a protectant against hyperoxia-induced oxidative stress (9). We employed this mutant strain rather than the intermittent intravenous administration of bilirubin as the latter fails to achieve sustained elevations in plasma bilirubin levels (Nath KA and Croatt AJ, unpublished observations).

The present studies thus unite prior investigative themes which, on the one hand, demonstrate the induction of HO-1 by ANG II (4, 15, 49), and on the other, the efficacy of bilirubin, a product of HO, to serve as an antioxidant (26). Our studies were also motivated by the mounting clinical evidence attesting to the vasoprotective and antioxidant effects of the HO system in general (7, 12, 17, 38, 39), and of its metabolite, bilirubin, in particular (11, 34, 46).

METHODS

Studies in vivo. Male Gunn and control Wistar rats were obtained from Harlan (Indianapolis, IN), and all experiments were conducted on age-matched rats 6 to 8 wk old. These rats were fed standard rat chow and given tap water ad libitum. We confirmed hyperbilirubinemia in the Gunn rat from spectrophotometric measurements of plasma bilirubin, performed as previously described (26): plasma levels of bilirubin were 4.7 ± 0.9 and 117.9 ± 9.8 μM in the control (n = 7) and Gunn (n = 9) rat, respectively. Such marked increments in plasma bilirubin reflect increases in unconjugated bilirubin. Our studies were approved by our Institutional Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

As described in our prior study (15), ANG II (0.5 mg·kg body wt⁻¹·day⁻¹, A-9525, Sigma, St. Louis, MO), dissolved in lactated Ringer solution, or vehicle (lactated Ringer solution) alone was administered by osmotic minipumps (model 2004, Alzet, Palo Alto, CA) to control and Gunn rats. The osmotic minipumps were implanted subcutaneously via a midscapular incision in rats anesthetized with Methohexital (50 mg/kg body wt ip). The basic study design consisted of four groups of rats: control rats receiving ANG II (n = 7), body wt: 169 ± 3 g), Gunn rats receiving ANG II (n = 9, body wt: 157 ± 2 g), control rats receiving vehicle (n = 6, body wt: 170 ± 9 g), and Gunn rats receiving vehicle (n = 6, body wt: 159 ± 5 g). One week before pump placement, systolic blood pressures and creatinine clearance were determined, and these indexes were determined at weekly intervals following the placement of the osmotic minipumps.

Systolic blood pressures were obtained by tail cuff plethysmography, as described in our prior study (15). Plasma and urinary concentrations of creatinine were determined by the Jaffé method using a Beckman Creatinine Analyzer II (Beckman Instruments, Fullerton, CA) (26). After 4 wk, rats in all four groups were killed. In these and additional cohorts, aortas were harvested for studies of vascular reactivity and the measurement of tetrahydrobiopterin (BH₄) and dihydrobiopterin (7,8-BH₂). We also determined the production of superoxide anion by aortic segments harvested from control Wistar rats subjected to the administration of either ANG II or vehicle, as described above, and the effect of bilirubin added in vitro on such generation of superoxide anion.

Vascular reactivity. Vascular reactivity was assessed in isolated aortic rings by methods described in detail in prior publications (13, 14, 33). In brief, isolated aortic rings (4-mm length) were connected to a force transducer for recording of isometric force and placed in an organ bath filled with 25 ml modified Krebs-Ringer solution (composition in mM: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, 0.026 calcium EDTA, and 11.1 glucose, pH 7.4), maintained at 37°C, and bubbled with a gaseous mixture of 94% O₂-6% CO₂. Endothelium-dependent relaxations in response to ace

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Left: systolic blood pressure (SBP) in ANG II-infused control rats (○, n = 7) and ANG II-infused Gunn rats (●, n = 9) at weekly intervals after the initiation of ANG II infusion by osmotic minipump. Right: changes in SBP in ANG II-infused control rats (open bars, n = 7) and ANG II-infused Gunn rats (filled bars, n = 9) at weekly intervals following the initiation of ANG II infusion by osmotic minipump. Statistical analysis was performed using ANOVA with Bonferroni’s test. *P < 0.05 at that time point. NS, not significant.
Angiotensin II (ANG II) infusion by osmotic mini-pumps was used in control rats to maintain arterial blood pressure near baseline levels before the initiation of ANG II infusion. However, 1 and 2 wk after the initiation of ANG II, systolic blood pressure and the increment in blood pressure from baseline values were significantly greater in ANG II-infused Gunn rats compared with ANG II-infused control rats during both systolic hypertension and diastolic hypertension phases. Nevertheless, both systolic hypertension and diastolic hypertension in Gunn rats were not different in ANG II-infused control rats and ANG II-infused Gunn rats, respectively. Over the course of the study, and unlike the ANG II-infused groups, no significant changes in systolic blood pressures in the vehicle-infused control and vehicle-infused Gunn rats did not increase (Table 1).

Glomerular filtration rate (GFR) tended to decrease in the control rat subjected to ANG II but remained relatively constant in the ANG II-infused Gunn rat (Fig. 2). The changes in GFR between the ANG II-infused control rat and ANG II-infused Gunn rat were statistically different at 4 wk after the administration of ANG II (Fig. 2).

Vascular reactivity in response to endothelium-dependent (acetylcholine) and endothelium-independent (DEA NONOate) vasodilators was also assessed in aortic rings in control and Gunn rats subjected to ANG II or vehicle. Acetylcholine-elicted vasorelaxation was significantly impaired in ANG II-infused control rats (Fig. 3, left) but was not impaired in the ANG II-infused Gunn rat (Fig. 3, right); dose-response curves in vehicle-infused control and vehicle-infused Gunn rats were not different. There was a tendency for an impairment in DEA NONOate-induced vasorelaxation in the ANG II-infused control rat (P = 0.055; Fig. 4, left) but not in the ANG II-infused Gunn rat (Fig. 4, right); dose-response curves in vehicle-infused control rats and vehicle-infused Gunn rats were not different. Thus the chronic administration of ANG II impairs endothelium-dependent relaxation and tends to impair endothelium-independent relaxation in the control but not in the Gunn rat.
To determine whether these changes in blood pressure in vivo as well as altered vascular reactivity in vitro were associated with changes in cellular redox in the vessel wall, we employed the redox-sensitive marker BH$_4$/BH$_2$. Absolute values for BH$_4$ and 7,8-BH$_2$ in each of the four groups are shown in Table 2, and the ratio, BH$_4$/BH$_2$, is displayed in Fig. 5. In the vehicle-infused control rat and the vehicle-infused Gunn rat, the BH$_4$/BH$_2$ ratios were not significantly different in the aortic segments (Fig. 5). ANG II infusion led to a significant decrement in the BH$_4$/BH$_2$ ratio in the aorta after 4 wk in control rats. However, in the Gunn rat, the decrement in this redox-sensitive index was markedly attenuated, and, indeed, was not significant in the ANG II-infused Gunn rat. Thus ANG II induces oxidative stress in the aorta of the control rat, whereas such effects are significantly mitigated in the Gunn rat.

To determine whether bilirubin effectively quenches superoxide anion generated in response to ANG II, studies were undertaken in vitro wherein aortic rings from vehicle-infused or ANG II-infused rats were exposed to bilirubin, and the generation of superoxide anion was determined. As shown in Fig. 6, superoxide anion generation almost doubled in rats in which ANG II was infused. The exposure of these aortic rings to increasing concentrations of bilirubin significantly decreased the generation of superoxide anion. Indeed, the generation of superoxide anion by aortic rings in vitro was preserved to a greater extent in rings harvested from ANG II-infused Gunn rats compared with rings taken from ANG II-infused control rats. Thus, based on pressor responses in vivo coupled with vascular reactivity in vitro, the hyperbilirubinemic Gunn rat is resistant to the vasoconstrictive effects of ANG II.

Because oxidative stress contributes to the pressor effects of ANG II (15, 25, 36, 40, 43, 49), we examined whether attenuation in oxidative stress accompanied this resistance of the Gunn rat to the pressor effects of ANG II. We employed the BH$_4$/BH$_2$ ratio as a marker of oxidative stress as this ratio provides a sensitive index for oxidative stress, in part, because of the susceptibility of BH$_4$ to oxidative attack (21–23, 45); additionally, changes in the BH$_4$/BH$_2$ ratio may offer insights regarding the pathophysiological effects of ANG II. In the vehicle-infused control rat and the vehicle-infused Gunn rat, the BH$_4$/BH$_2$ ratios were not significantly different in the aortic segments. This lack of difference between these groups likely reflects the paucity of ambient oxidative stress in the basal,

**DISCUSSION**

Our findings demonstrate that in response to chronic administration of ANG II, the rise in systemic blood pressure in hyperbilirubinemic Gunn rats was markedly blunted and, indeed, was reduced to 50% of that observed in the ANG II-infused control group. In addition to this attenuation in the pressor effects of ANG II in vivo, the Gunn rat exhibited less impairment in vascular reactivity as studied in vitro. For example, endothelium-dependent vasorelaxation in aortic rings was preserved to a greater extent in rings harvested from ANG II-infused Gunn rats compared with rings taken from ANG II-infused control rats. Thus, based on pressor responses in vivo coupled with vascular reactivity in vitro, the hyperbilirubinemic Gunn rat is resistant to the vasoconstrictive effects of ANG II.

**Fig. 3.** Left: acetylcholine-dependent vasorelaxation in aortic rings harvested from ANG II-infused control rats (●, n = 8) and vehicle-infused control rats (○, n = 6). The dose-response curves of the different groups were compared by ANOVA for repeated measurements with Bonferroni’s test. *P < 0.05 between the 2 dose-response curves. Right: acetylcholine-dependent vasorelaxation in aortic rings harvested from ANG II-infused Gunn rats (●, n = 8) and vehicle-infused Gunn rats (○, n = 6).

**Fig. 4.** Left: DEA NONOate-dependent vasorelaxation in aortic rings harvested from ANG II-infused control rats (●, n = 8) and vehicle-infused control rats (○, n = 6). The dose-response curves of the different groups were compared by ANOVA for repeated measurements with Bonferroni’s test. *P = 0.055. Right: DEA NONOate-dependent vasorelaxation in aortic rings harvested from ANG II-infused Gunn rats (●, n = 8) and vehicle-infused Gunn rats (○, n = 6).
unstressed state and is consistent with prior studies demonstrating no differences in oxidant indexes (such as TBARS) in the basal, unstressed state in control and Gunn rats (9). However, in response to ANG II, the BH4/BH2 ratio fell quite significantly in aortic segments in control rats, whereas in Gunn rats the reduction in this ratio in response to ANG II was blunted. Thus, using this marker, oxidative stress incurred by ANG II is mitigated in the Gunn rat, the latter also exhibiting a blunted pressor response to ANG II.

To determine the mechanism whereby oxidative stress may be attenuated, we drew on the recognized capacity of bilirubin to serve as an antioxidant. We demonstrate that aortic rings from ANG II-infused rats exhibited a marked increase in production of superoxide anion, and this was reduced in a dose-dependent fashion by bilirubin. Indeed, the generation of superoxide anion by aortic rings harvested from vehicle-infused rats exhibited a marked increase in ANG II, as observed in our present studies, is a novel finding with regard to the vascular effects of ANG II. Additionally, this finding raises the possibility that enhanced generation of superoxide anion by ANG II may be aided by other sources in addition to NADPH oxidase. BH4, a necessary cofactor for endothelial nitric oxide synthase (eNOS), enables NOS to oxidize arginine to citrulline with attendant generation of NO (21–23, 45). A diminished ratio of BH4/BH2 may lead to an uncoupling of eNOS such that oxidation of arginine to citrulline is less effectively achieved by eNOS, and less NO is thereby produced; concomitantly, increased amounts of superoxide anion are generated (21–23, 45). An uncoupling of eNOS thus limits the supply of NO and provides a source of superoxide anion. Such an alteration in eNOS activity is doubly disadvantageous to the endothelium: the diminished amounts

Table 2. BH4, 7, 8-BH2, and total biopterin content of aortas from vehicle-infused control and Gunn rats and ANG II-infused control and Gunn rats

<table>
<thead>
<tr>
<th></th>
<th>BH4</th>
<th>7,8-BH2</th>
<th>Total Biopterin, pmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-infused control (n = 4)</td>
<td>1.7±0.3</td>
<td>0.1±0.02</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>Vehicle-infused Gunn (n = 5)</td>
<td>3.2±0.3*</td>
<td>0.25±0.03</td>
<td>3.5±0.3‡</td>
</tr>
<tr>
<td>ANG II-infused control (n = 4)</td>
<td>2.6±0.3</td>
<td>0.49±0.11†</td>
<td>3.1±0.4‡</td>
</tr>
<tr>
<td>ANG II-infused Gunn (n = 6)</td>
<td>2.7±0.3</td>
<td>0.33±0.03‡</td>
<td>3.1±0.3‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. BH4, tetrahydrobiopterin (pmol/mg protein); 7, 8-BH2, dihydrobiopterin (pmol/mg protein). *P < 0.05 vs. BH4 values in vehicle-infused control rat. †P < 0.05 vs. 7, 8-BH2 values in vehicle-infused control rat. ‡P < 0.05 vs. total biopterin content in vehicle-infused control rat (statistical analysis by ANOVA with Bonferroni’s test).
of NO may lead to endothelial dysfunction, whereas superoxide anion, by combining with NO to form peroxynitrite, may further impair the already diminished availability of NO. An added consideration is the fact that BH4 is vulnerable to oxidative attack and can be depleted in states of oxidative stress (21–23, 45). Based on these considerations, and the fact that oxidative stress can lower the BH4/BH2 ratio, we speculate that oxidative stress, originating from ANG II-induced activation of NADPH oxidase, diminishes the BH4/BH2 ratio, thereby transforming eNOS from an NO-generating enzyme to one that produces superoxide anion: in this setting, eNOS, we speculate, may now contribute to oxidative stress.

In our studies, the hyperbilirubinemic Gunn rat exhibited greater preservation of endothelial function and greater preservation in the BH4/BH2 ratio following the administration of ANG II. Given the remarkable efficacy of bilirubin in scavenging superoxide anion generated by aortic rings, we speculate that the increased amounts of bilirubin in the systemic circulation in the Gunn rat scavenge superoxide anion generated in response to ANG II: the BH4/BH2 ratio is thus preserved, eNOS is less uncoupled, and endothelium-dependent relaxation less impaired. However, other mechanisms may underlie this greater preservation of the BH4/BH2 ratio in the hyperbilirubinemic rat. For example, bilirubin may proffer chemical stabilization of BH4 as has been shown for another endogenous metabolite, vitamin C, through such a mechanism, vitamin C augments tissue levels of BH4 and increases eNOS activity (6, 14). It is also conceivable that bilirubin may affect enzymes, such as GTP-cyclohydrolase (21, 22), that are critically involved in the pathways that synthesize BH4.

Enzymes such as GTP-cyclohydrolase that are involved in biopterin synthesis are inducible by cytokines and proinflammatory states (21, 22). It is possible that the recognized proinflammatory effects of ANG II may underlie the increase in total biopterin content observed in the aorta when ANG II is administered to control rats (Table 2). It is notable, however, that total biopterin content fails to increase in Gunn rats subjected to ANG II (Table 2); this lack of an increase in total biopterin levels in response to ANG II in the Gunn rat may reflect the anti-inflammatory effects of bilirubin, the latter abundantly described in other settings (12, 16, 27, 39).

Other described actions of bilirubin may be relevant to the effects we observed in vivo using the hyperbilirubinemic Gunn rat. For example, bilirubin inhibits PKC activation (5), the latter contributing to ANG II-induced vasoconstriction (30). Bilirubin can also directly inhibit the NADPH oxidase enzyme in cell-free systems (24), and thus direct inhibitory effects of bilirubin on the activity of NADPH oxidase, in addition to scavenging superoxide anion, may account for the mitigating effect of bilirubin on oxidative stress. Although, clearly, other actions of bilirubin may contribute to the resistance observed in the Gunn rat to the pressor effects of ANG II, our findings add to the developing body of literature attesting to the beneficial effects of bilirubin. For example, bilirubin protects against oxidative stress in renal and other cell types (26, 37, 47) and reduces oxidative stress-induced injury in vivo in the liver, the nervous system, the gastrointestinal tract, and other tissues (16, 26, 27, 48). Bilirubin protects against cardiac injury in the isolated, perfused myocardium (8), and, indeed, bilirubin has been proposed as an additive to preservation solutions used in organ transplantation (20).

A number of studies attest to HO in general (7, 12, 17, 38), and bilirubin in particular (11, 34, 46), as protectants against cardiovascular disease. For example, certain polymorphisms in the HO-1 gene that lead to increased HO activity are associated with a lower risk of cardiovascular disease (7, 38). Interestingly, in epidemiological studies of cardiovascular disease, patients with higher bilirubin levels have a lower incidence of cardiovascular disease (11, 34). Finally, patients with Gilbert’s disease (in whom there is unconjugated hyperbilirubinemia due to a deficiency of UDPGT) exhibit a reduced risk for cardiovascular disease (46). Because atherosclerosis may be associated with oxidative stress, and with systemic as well as local activation of the renin-angiotensin system (40, 43), it is possible that the diminished risk for cardiovascular disease in patients with hyperbilirubinemia may reflect the antioxidant effects of bilirubin with the attendant mitigation of oxidative stress, induced by ANG II and other mechanisms.

We wish to point out that the model of ANG II-induced hypertension used in the present study achieves marked elevations in plasma levels of ANG II (10). Such marked elevation in ANG II may swamp any differences in plasma levels of ANG II that may exist in the basal state in control and Gunn rats. Nonetheless, studies of the renin-angiotensin system in control and Gunn rats, in the basal state and following the administration of ANG II, would be of interest.

In summary, we demonstrate that the hyperbilirubinemic Gunn rat exhibits resistance to the pressor effects of ANG II in vivo, resistance to ANG II-induced impairment in vascular reactivity in vitro, and attenuation of oxidative stress induced by ANG II; we speculate that these effects may arise, at least in part, from the quenching of oxidative stress by bilirubin. We suggest that our observations are relevant to, and may offer insights into, the mounting clinical evidence attesting to the vasoprotective and antioxidant effects of the HO system in general, and of its metabolite, bilirubin, in particular.

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