Antihypertensive properties of a nitric oxide-releasing naproxen derivative in two-kidney, one-clip rats

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Received 9 November 1999; accepted in final form 10 February 2000

THE MAJOR LIMITATION to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is their ability to cause damage and bleeding in the upper gastrointestinal tract (23). Another significant limitation to the use of these drugs pertains to their ability to interfere with the efficacy of antihypertensive therapies (7, 8) and, in some patients, to directly exacerbate hypertension (28).

A recent approach to reduce NSAID-induced gastrointestinal toxicity involves the derivatization of NSAIDs to incorporate a nitric oxide (NO)-releasing moiety (24). These compounds, referred to as “NO-NSAIDs,” exhibit comparable or even improved anti-inflammatory and analgesic activities in comparison to the parent drugs (4, 25, 27). Moreover, we recently reported that daily administration of an NO-releasing derivative of naproxen, HCT-3012, to rats for a period of 4 wk significantly attenuated the increase in blood pressure (BP) induced by addition of an NO synthase inhibitor to the drinking water (18). On the other hand, an equimolar dose of naproxen significantly potentiated the hypertension in these rats. These differences between naproxen and HCT-3012 occurred despite the fact that both compounds exhibited similar inhibitory effects on cyclooxygenase (COX) activity.

The ability of HCT-3012 to reduce BP in rats with Nω-nitro-L-arginine methyl ester (L-NAME)-induced hypertension may have been related to the hypersensitivity of vascular smooth muscle to exogenous NO that develops when NO synthase is chronically suppressed (8, 10). Thus the beneficial effects of NO-NSAIDs in the L-NAME-induced hypertension model may have simply been due to delivery of NO to an NO-deficient vasculature. To further characterize the pressor effects of NSAIDs in hypertensive rats, and the possibility that an NO-NSAID would lack pressor effects, we tested these compounds in another model of hypertension that does not involve direct manipulation of NO synthesis. In this study, therefore, we examined the effects of treatment with naproxen or HCT-3012 in the “two kidney, one clip” (2K,1C) renovascular hypertension model in rats. In addition, the in vitro effects of these compounds on contractility of aortic rings obtained from either normotensive or hypertensive rats were examined.

METHODS

Animals. Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, Quebec, Canada). The rats were housed in polypropylene cages in groups of two or three rats per cage and received laboratory chow ad libitum. After a 1-wk period of acclimatization, the systolic BP of each rat was measured (see Blood pressure measurement).

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**measurement**, after which the surgical procedure for induction of renovascular hypertension was performed.

2K,1C renovascular hypertension. After the rats were anesthetized by exposure to halothane vapor (5% in oxygen), a 3-cm retroperitoneal flank incision was performed under sterile conditions. The left kidney was exposed and the renal artery was carefully dissected free of the renal vein. The renal artery was then partially occluded by placing a silver clip with an internal diameter of 0.20 mm on the vessel. The wound was closed with a running 3-0 silk suture, and the rats were then randomly assigned to one of three treatment groups (n = 8 per group), as described in Treatments. Sham-operated rats (n = 8) underwent identical surgical procedures, except that a clip was not applied to the renal artery.

**Treatments.** Two weeks after the renal artery clipping procedure, the 2K,1C rats (subsequently referred to as “hypertensive rats”) were treated daily with vehicle (1 ml/kg po), naproxen (10 mg/kg po), or an equimolar dose of HCT-3012 (14.5 mg/kg po) for 2 wk. Sham-operated rats received vehicle (1 ml/kg). The rats consisted of 0.5% carboxymethylcellulose as 0.9% NaCl (95:5 ratio, vol:vol). Naproxen and HCT-3012 were dissolved in this solution at 10 and 14.5 mg/ml, respectively.

**Blood pressure measurement.** Weekly measurement of systolic arterial pressure was performed by the tail-cuff method (20). The rats were conscious and only mildly restrained during the measurements, which were always made between 1300 and 1500. The BP measurements were considered valid only when three consecutive readings did not differ by more than 5 mmHg. The mean of the three measured values was then recorded. The individual performing the measurements was unaware of the treatments the rats had received.

**Thromboxane B2 synthesis.** At the end of the 2-wk treatment period, 2 h after the last dose of vehicle, naproxen, or HCT-3012, the rats were anesthetized with pentobarbital sodium, and the synthesis of thromboxane B2 (TxB2) by whole blood was determined as previously described (24).

**Serum nitrite and nitrate concentrations.** Blood sample aliquots (2 ml) were allowed to clot at room temperature. The sera were separated by 10 min of centrifugation at 2,000 g and kept at −20°C until analyzed by high-performance liquid chromatography (17) for their nitrite and nitrate concentrations.

**Plasma renin activity.** Blood sample aliquots (1 ml) were collected in precooled tubes containing 0.8 mg of EDTA, and the plasma was immediately separated by centrifugation (2,000 g during 10 min at 4°C) and kept at −20°C until analyzed. Plasma renin activity was estimated by the generation of angiotensin I from endogenous angiotensinogen at pH 7.4 and 37°C during 4 h. Angiotensin I concentrations were measured by radioimmunoassay.

**In vivo response to sodium nitroprusside.** A separate group of rats was subjected to either left renal artery clipping or sham operation. Four weeks after the surgical procedure, the rats were anesthetized by an injection of pentobarbital sodium (65 mg/kg ip) and placed on a heating blanket to maintain their body temperature at 37°C. The left carotid artery was catheterized with polyethylene-10 tubing and connected to a pressure transducer for continuous monitoring of systolic and diastolic arterial pressure. The right femoral vein was cannulated with a polyethylene-50 catheter through which increasing doses of sodium nitroprusside (0.8–16 μg/kg) were injected. The effects on mean arterial pressure (MAP) were recorded.

**Effects of acute pretreatment with NSAIDs on the response to endothelin-1.** Normotensive rats were cannulated (see In vivo response to sodium nitroprusside), and the changes in MAP after the intravenous administration of increasing doses of endothelin-1 (ET-1, 160–800 pmol/kg) were recorded. The rats then received a single dose of either naproxen (10 mg/kg ip) or HCT-3012 (14.5 mg/kg ip), and after 1 h the dose-response curves for ET-1 were repeated.

**In vitro vascular response of aortic rings.** Thoracic aortae obtained from hypertensive or control rats were cleaned of all connective tissue and cut into rings of 3–4 mm length. Each ring was mounted under 2 g passive tension in a 25-ml organ bath containing a physiological salt solution maintained at 37°C and bubbled with 95% O2-5% CO2. Isometric tension was recorded with a force-displacement transducer (Grass FT 03) coupled to a Grass polygraph model 7D. The physiological solution used had the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 12.5 NaHCO3, and 11.1 glucose. Tissues (endothelium-intact preparations) were routinely allowed to equilibrate for 1 h before the experiments were started. After the equilibration period, cumulative concentration-response curves to phenylephrine were carried out for all the rings. Glycerol trinitrate (GTN) or HCT-3012 was then added cumulatively to each muscle preparation to elicit relaxation, and the concentration-response curves were constructed. After a 15-min incubation period with 10 μM of either GTN or HCT-3012, the concentration-response curves to phenylephrine were repeated. In all experiments, each ring preparation was exposed to a single NO donor, and from each aorta, one ring served as the control by being exposed only to phenylephrine.

The in vitro experiments outlined above were also performed using aortic rings obtained from rats that had received 400 mg/l 1-NAME (Sigma Chemical, St. Louis, MO) in the drinking water for the previous 2 wk. Control rats received tap water. Based on the individual daily liquid intake, this 1-NAME concentration resulted in a dose of −45 mg kg−1 day−1.

**Statistical analysis.** All data are shown as means ± SE. Differences among groups were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Values of P < 0.05 were considered significant.

**Materials.** Naproxen, sodium nitroprusside, and ET-1 were obtained from Sigma Chemical. HCT-3012 was provided by NicOx (Sophia Antipolis, France). GTN was obtained from Omega (Montreal, Canada). Kits for measurement of TxB2 were obtained from Caymen Chemical (Ann Arbor, MI). The radioimmunoassay kit for angiotensin I was obtained from Peninsula (San Carlos, CA). All other supplies and reagents were obtained from Fisher Scientific (Edmonton, Alberta, Canada).

**RESULTS**

As shown in Fig. 1, the partial occlusion of the left renal artery induced a sustained increase in BP values during the first 2 wk following the clipping procedure. With daily naproxen treatment during the 2 wk that followed, a significantly more severe hypertension was observed, in comparison to the rats receiving vehicle. Over the 2-wk period of treatment, the BP was increased by ~24 mmHg over that in the vehicle-treated rats. In contrast, rats treated daily with HCT-3012 exhibited significantly lower BP levels than those treated with either vehicle or naproxen. The mean decrease in BP relative to the vehicle-treated rats was ~17 mmHg, and the decrease relative to the naproxen-treated rats was ~41 mmHg. Throughout the 4-wk
study the sham-operated animals had BP levels similar to those recorded before the surgical procedure.

HCT-3012-treated hypertensive rats exhibited significantly higher serum nitrate levels than any of the other groups ($P < 0.001$), although circulating nitrite concentrations were not affected by any of the treatments (Fig. 2A). Additionally, no statistically significant differences were observed between hypertensive rats and the sham-operated controls in terms of either systemic nitric oxide production (Fig. 2A) or whole blood Tx$\text{B}_2$ synthesis (Fig. 2B). However, Tx$\text{B}_2$ production in both the naproxen- and HCT-3012-treated rats was significantly inhibited ($>80\%$) relative to that observed in the vehicle-treated group ($P < 0.001$).

No statistically significant differences in plasma renin activity were observed among the four groups at the end of the 4-wk study period: sham, $4.2 \pm 1.1 \text{ ng} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$; 2K,1C + vehicle, $4.0 \pm 1.2 \text{ ng} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$; 2K,1C + naproxen, $2.5 \pm 0.5 \text{ ng} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$; and 2K,1C + HCT-3012, $4.5 \pm 0.5 \text{ ng} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$ ($P = 0.37$).

The antihypertensive effects of HCT-3012 were further evaluated in anesthetized rats. Initially we characterized the responsiveness of control and hypertensive rats to a standard NO donor. Figure 3A shows that the absolute fall in MAP caused by the intravenous administration of sodium nitroprusside was greater in the hypertensive rats than in controls. However, because of the significant difference in basal MAP values between the two groups ($179 \pm 9 \text{ mmHg}$ in 2K,1C vs. $123 \pm 4 \text{ mmHg}$ in controls, $P < 0.001$), the response to sodium nitroprusside, as a percentage of basal MAP, remained unchanged (Fig. 3B).

The effects of pretreatment with either naproxen or HCT-3012 on the pressor response to ET-1 in normotensive rats are depicted in Fig. 4. Figure 4A shows that the hypertensive effect of ET-1 (measured as the maximum of the late phase of the response) was potentiated by naproxen pretreatment, and HCT-3012 pretreatment was devoid of any significant effect on this response. In the same way, the early hypotensive phase of the response to all concentrations of ET-1 was significantly attenuated by naproxen pretreatment, without any statistically detectable effect of HCT-3012 pretreatment on this response (Fig. 4B).

Figure 5 shows the in vitro vasorelaxant activities of HCT-3012 and GTN in phenylephrine-precontracted aortic rings obtained from hypertensive and control rats (phenylephrine was used at concentrations ranging from 1 to 10 $\mu$M to produce 70% of maximal contraction). Vessels from hypertensive rats did not differ significantly from those from controls in terms of the relaxation response to GTN (the concentration values yielding the half-maximal relaxation response, $pEC_{50}$, were $8.37 \pm 0.09$ and $8.33 \pm 0.06$, respectively), and no alteration in the percentage achieving the maximal relaxation response ($E_{max}$) was observed at the $10^{-5}$ M concentration ($E_{max}$ values of 79.9 ± 4.6% and 70.6 ± 9.0% for hypertensive and sham, respectively). From the $pEC_{50}$ values, it can be seen that HCT-3012 relaxed the aortic rings with a potency approximately two orders of magnitude lower than that of GTN.

![Fig. 1. Effects of naproxen and HCT-3012 on systolic blood pressure in renovascular hypertensive two-kidney, one-clip (2K,1C) rats: sham-operated group (●); vehicle-treated 2K,1C group (○); naproxen-treated 2K,1C group (■); and HCT-3012-treated 2K,1C group (□). Arrow indicates the point at which treatment with test compounds was initiated. $**P < 0.01$, ***$P < 0.001$ vs. vehicle-treated 2K,1C group, ###$P < 0.001$ vs. HCT-3012 group.](http://ajpheart.physiology.org/)

![Fig. 2. A: nitrate (NO$_3^-$) and nitrite (NO$_2^-$) concentrations measured in serum samples from rats with 2K,1C-induced hypertension treated with vehicle, naproxen, or HCT-3012, and in sham-operated rats. ***$P < 0.001$ vs. remaining groups. B: thromboxane B$_2$ (Tx$\text{B}_2$) synthesis by whole blood samples from vehicle-, naproxen-, or HCT-3012-treated hypertensive rats and sham-operated rats. ###$P < 0.01$ and ###$P < 0.001$ vs. naproxen-treated rats, $$$P < 0.001$ vs. HCT-3012-treated rats.](http://ajpheart.physiology.org/)
Phenylephrine contracted aortic rings from sham-operated and renovascular hypertensive rats in a dose-dependent manner, with pEC₅₀ values of 6.10 ± 0.03 and 6.82 ± 0.05, respectively (P < 0.001; Fig. 6). The preincubation of the aortic rings from controls with either GTN or HCT-3012 induced similar rightward shifting of the phenylephrine contraction curves (pEC₅₀ values of 5.78 ± 0.04 and 5.69 ± 0.07 for GTN and HCT-3012, respectively). However, although the preincubation of the aortic rings with 10⁻⁵ M GTN did not cause any alteration of the maximal phenylephrine-induced contraction, preincubation with HCT-3012 led to a slight (but still significant) decrease of the maximal contraction to phenylephrine (Eₘₐₓ value of 94.2 ± 2.9%, P < 0.05; Fig. 6A). On the other hand, in the case of the vascular tissue from hypertensive rats, preincubation with either of the NO donors led to significant loss of both efficacy (Eₘₐₓ values of 61.3 ± 2.4% for GTN and 74.5 ± 2.4% for HCT-3012; P < 0.001 vs. control) and potency (pEC₅₀ values of 5.96 ± 0.10 for GTN and 6.10 ± 0.09 for HCT-3012; P < 0.001 vs. control) of phenylephrine-induced vasoconstriction.

Figure 7 shows the in vitro vasorelaxant activities of HCT-3012 and GTN in phenylephrine-precontracted aortic rings obtained from either control or l-NAME-treated rats. Tissues obtained from rats with l-NAME-induced hypertension were more sensitive to the relaxant activity of GTN than those obtained from control animals (pEC₅₀ values of 7.76 ± 0.03 vs. 8.17 ± 0.06 for control and l-NAME groups, respectively; P < 0.01) with no significant alteration in the maximal response (Eₘₐₓ value of 100% for both groups). In the case of HCT-3012, phenylephrine-precontracted aortic rings from l-NAME-treated rats showed a slightly diminished response to this compound compared with that observed in tissues obtained from control animals (pEC₅₀ values of 6.71 ± 0.14 vs. 7.07 ± 0.07 for l-NAME and control, respectively; P < 0.05). However, no significant differences were observed in terms of maximal relaxation to HCT-3012 between the experimental groups (Eₘₐₓ values of 75.7 ± 3.8% and 78.8 ± 3.0% for control and l-NAME-treated rats, respectively).
Figure 8 shows the phenylephrine-induced contraction curves obtained with aortic rings from either control or L-NAME-treated rats. The respective pEC\textsubscript{50} values were 6.49 \pm 0.04 and 7.19 \pm 0.03 (P < 0.001). Preincubation of aortic rings obtained from control animals with 10^{-5} M GTN or HCT-3012 for 15 min induced small but significant decreases of the pEC\textsubscript{50} value for phenylephrine-induced contraction (6.17 \pm 0.06 and 6.13 \pm 0.04 for GTN and HCT-3012, respectively). However, although the preincubation of the aortic rings with GTN did not cause any significant...
alteration of the maximal phenylephrine-induced contraction ($E_{\text{max}}$ values of 100% and 97.5 ± 2.5% for control and GTN, respectively), preincubation with HCT-3012 led to a decrease of the maximal contractile response to phenylephrine ($E_{\text{max}}$ of 84.2 ± 5.0%, $P < 0.001$; Fig. 8A). In the case of vascular tissue obtained from L-NAME-treated rats, the preincubation with any of the NO donors led to a significant loss in the efficacy of phenylephrine-induced vasoconstriction ($E_{\text{max}}$ values of 61.3 ± 4.3% for GTN and 43.6 ± 3.2% for HCT-3012; $P < 0.001$ vs. control), and the dose-response curves to phenylephrine were significantly shifted to the right ($pE_{50}$ values of 6.36 ± 0.09 for GTN and 6.47 ± 0.10 for HCT-3012; $P < 0.001$ vs. control).

**DISCUSSION**

The results of the present study demonstrate that the hypertensive state produced in rats by partial occlusion of the left renal artery is significantly exacerbated by naproxen, consistent with clinical reports that NSAIDs can exacerbate hypertension in some groups of patients (11). On the other hand, a NO-releasing derivative of naproxen, despite markedly suppressing cyclooxygenase activity, was capable of significantly reducing BP in the renovascular hypertensive (2K,1C) rats. Muscará et al. (18) previously observed a significant antihypertensive effect of HCT-3012 in rats made hypertensive through addition of L-NAME to the drinking water. In that study, however, HCT-3012 was administered before the establishment of a hypertensive state, and it was possible that HCT-3012 reduced BP simply by supplying NO to an NO-depleted vasculature. It is also important to note that in the earlier study (18), HCT-3012 administration at the same dose as used in the present study did not significantly decrease BP in normotensive rats.

Systemic NO production was unaltered in 2K,1C rats compared with controls and was not altered by treatment with naproxen. However, HCT-3012 treatment resulted in significant increases in serum nitrate, consistent with the NO-donating properties of this compound and other NO-NSAIDs (4, 24, 25, 27). At first sight, one might conclude that the ability of HCT-3012 to reduce BP in the 2K,1C rats is simply related to the vasodilator effects of NO released from this compound. Interestingly, however, NO-NSAIDs have been shown to be devoid of hypotensive effects in normotensive rats, despite producing similar increases in serum nitrate (18, 24, 25). Furthermore, the 2K,1C rats did not exhibit an increased responsiveness to the hypotensive effects of sodium nitroprusside. As shown in Fig. 3, despite sodium nitroprusside causing a more pronounced decrease in MAP in 2K,1C rats than in sham-operated controls, these responses were identical when expressed as a percentage of basal MAP. The in vitro dose-dependent relaxation curves to GTN and HCT-3012 further support this conclusion. Aortic rings prepared from either 2K,1C or sham-operated rats relaxed to these NO donors in an indistinguishable manner. In contrast, vascular hypersensitivity to exogenous NO has previously been demonstrated in vitro under conditions of either endogenous NO synthesis inhibition or after removal of the endothelium (10, 16).

In the present study we demonstrated that GTN relaxed aortic rings obtained from L-NAME-treated rats at lower concentrations than those required for tissues obtained from normotensive control rats. Interestingly, these effects were not seen with HCT-3012, which dose dependently relaxed aortic rings from L-NAME-treated rats with a $pE_{50}$ value slightly lower than that observed in vessels from normotensive rats.

Increased sympathetic activity has been observed in hypertensive rats (19) and humans (12) and seems to be due to higher levels of norepinephrine release and the greater affinity of this mediator for the $\alpha$-adrenergic receptor (13). In the present study we observed hyperreactivity to phenylephrine of aortic rings from 2K,1C rats in comparison to those obtained from sham-operated rats. The incubation with either GTN or HCT-3012 caused a modest attenuation of the response to phenylephrine in the case of control-derived aortae, although highly significant decreases of both efficacy and potency were observed in tissues from hypertensive rats. Interestingly, a very similar pattern was observed in tissues obtained from rats under chronic NO synthesis inhibition (Fig. 8), despite the etiological differences between these two models of hypertension. The contribution of the sympathetic nervous system to the hypertension induced by NO-synthesis blockade has already been demonstrated in vivo (9, 29) and in several vascular preparations in vitro (15, 21). The effects of NO on the vascular responsiveness to phenylephrine do not seem to be related to the modulation of intracellular calcium release through phosphatidylinositol turnover (20) but depend on the activation of triethylammonium-sensitive potassium channels (2). Independent of the molecular mechanisms involved, the observed attenuation by NO donors (and in particular, HCT-3012) of the vasoconstrictor response of vascular tissue from hypertensive animals to phenylephrine in vitro could, at least partly, account for the observed effects on BP in vivo.

Acute pretreatment with naproxen potentiated the late hypertensive phase of the response to ET-1 and attenuated the early hypotensive phase, although HCT-3012 pretreatment did not interfere with the pressor response to ET-1 in vivo. The vasoconstrictor effects of ET-1 are mediated by the activation of both ET$_A$ (1) and ET$_B$ (3) receptors expressed in the vasculature. ET-1 acting on its ET$_A$-type receptor expressed on the endothelium causes the release of NO and PGI$_2$, which in turn relax vascular smooth muscle via intracellular increases in cGMP and cAMP, respectively (5). In this way, and as previously described (6), the modulating effects of prostaglandins on ET-1-induced vasoconstriction are blunted by naproxen via COX inhibition. Interestingly, HCT-3012 pretreatment did not significantly affect the pressor response to ET-1, despite suppressing COX activity by >80%. Thus it seems possible that the NO released by HCT-3012
could attenuate the NSAID-potentiated ET-1 vasoconstrictor response. The possibility that HCT-3012 modulates the actions of ET-1 at other sites involved in BP regulation cannot be ruled out. For example, the hemodynamic effects of ET-1 are of particular importance in the renal microcirculation, where the vasoconstriction/vasodilatation balance (through ETA and ETB receptors, respectively) regulates renal blood flow, glomerular filtration rate, and renin secretion (14). Fujihara et al. (7) reported the beneficial effects of a NO-releasing derivative of flurbiprofen on systemic BP and renal function in nephrotic rats.

Despite the 2K,1C hypertension model being characterized by activation of the renin-angiotensin system after the renal artery occlusion, we did not observe significant changes in plasma renin activity in the 2K,1C rats in comparison to the sham-operated controls. This may be explained by the fact that all the rats underwent a “low sodium” diet during the 16–20 h before being killed (they received no food and had double-distilled water to drink to minimize exogenous nitrate anion ingestion). It is possible that renin release was stimulated in sham-operated rats by the low sodium intake, thus masking differences with 2K,1C rats.

In summary, we conclude that in contrast to the exacerbation of renovascular hypertension by naproxen, a NO-releasing naproxen derivative, HCT-3012, was able to significantly reduce systemic BP. It is important, in this regard, to note that HCT-3012 markedly suppressed COX activity and has previously been shown to have similar or enhanced anti-inflammatory and analgesic activities to the parent drug (4). The beneficial effects of this compound may be mediated at least in part through modulation of the actions of ET-1 and through attenuation of the sympathetic control of blood vessel tone but not via a direct vasodilator action of the NO released from this compound. The beneficial effects of HCT-3012 on hypertension, along with its gastric-sparing properties, make this compound an attractive alternative to conventional NSAIDs for the treatment of inflammatory processes in hypertensive patients.

This work was supported by a grant from the Heart and Stroke Foundation of Canada. J. L. Wallace is a Medical Research Council of Canada Senior Scientist and an Alberta Heritage Foundation for Medical Research Scientist. M. N. Muscará is supported by a Merck Pharmacology Fellowship.

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