Cedrelopsis grevei induced hypotension and improved endothelial vasodilatation through an increase of Cu/Zn SOD protein expression

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Ralay Ranaivo, Hantamalala, Olivier Rakotoarison, Angela Tesse, Christa Schott, Adolphe Randriantssoa, Annelise Lobstein, and Ramaroson Andriantsitohaina. Cedrelopsis grevei induced hypotension and improved endothelial vasodilatation through an increase of Cu/Zn SOD protein expression. Am J Physiol Heart Circ Physiol 286: H775–H781, 2004. First published November 6, 2003; 10.1152/ajpheart.00584.2003.—This study was designed to investigate the cardiovascular consequences of oral administration of Cedrelopsis grevei (CG) in normotensive rats. Experiments were designed to investigate hemodynamic parameters in vivo as well as the consequences of CG treatment on the vasoconstriction response to norepinephrine and the vasorelaxant response to ACh ex vivo in isolated aortas and small mesenteric arteries (SMAs). Treatment of male Wistar rats with 80 mg/kg CG for 4 wk induced a progressive decrease in systolic blood pressure. In the aorta, CG did not significantly alter the response to norepinephrine despite the participation of the extraendothelial nitric oxide (NO)-induced hyporeactivity. In the SMA, contractile responses to norepinephrine were not modified by CG treatment even though it enhanced the participation of endothelial NO. Endothelium-dependent relaxation to ACh was increased in both the aorta and SMA from CG-treated rats. In the aorta from CG-treated rats, the mechanism involved superoxide dismutase (SOD)- and catalase-sensitive free radical production. The latter was associated with enhanced expression of Cu/Zn SOD and endothelial NO synthase. These results suggest that oral administration of CG produces a decrease in blood pressure in normotensive rats. This hemodynamic effect was associated with enhanced endothelium-dependent relaxation and an induction of Cu/Zn SOD and endothelial NO synthase expressions in the vessel wall. They also show subtle mechanisms that compensate for the increased participation of NO to maintain unchanged agonist-induced contractility. These data provide a pharmacological basis for the empirical use of CG against cardiovascular diseases.

blood pressure; vasoconstriction

CEDRELOPSIS GREVEI (CG) Baill (Meliaceae) is an endemic plant from the southern and western areas of Madagascar empirically used against pulmonary diseases such as cough, tuberculosis, and pneumonia. This plant is also used as an antiasthemic, anti-inflammatory, and antihypertensive drug (4, 5, 10).

Different classes of compounds have been isolated from CG including chromones, coumarins, triterpenoids, limonoids, and polyphenols (9, 13, 20, 27). These compounds are known to be biologically active and beneficial against different pathologies such as inflammation, cancer, and cardiovascular diseases (15, 19). Among these compounds, the most active are the molecules from the class of polyphenols and coumarins. Indeed, they have been reported to scavenge reactive oxygen species (ROS), inhibit oxidation of low-density lipoprotein, prevent platelet aggregation, induce vasorelaxation, and interact with the nitric oxide (NO) pathway (1, 2, 14, 16, 22, 23).

Very recently, we reported that a crude extract of CG was able to induce relaxation of the rat aorta in both the presence or absence of functional endothelium (24). The endothelium-dependent component of the response was completely prevented by NO synthase (NOS) and guanylyl cyclase inhibitors, suggesting involvement of the NO pathway. A bioguided fractionation study (24) from the original extract showed that some of the active compounds belong to the class of coumarins and polyphenols. However, no studies have investigated the cardiovascular effect of CG in vivo after an oral administration. Indeed, it is not known whether sufficient levels of active compounds might be reached in the circulation.

Therefore, the present study was aimed to investigate the effects of oral administration of CG on hemodynamic parameters in vivo. The vasoconstriction response to an agonist, norepinephrine (NE), and the vasorelaxant response to ACh were also investigated ex vivo in isolated aortic rings and small mesenteric arteries (SMAs). Expression of endothelial NOS (eNOS), inducible NOS (iNOS), and Cu/Zn superoxide dismutase (SOD) were also determined.

METHODS

This investigation conforms to Authorization No. 01918 given by the French government Department of Agriculture. Two groups of male Wistar rats (12–14 wk old) were treated daily for 4 wk by an intragastric gavage of either vehicle (control) or 80 mg/kg·day1 extract of CG trunk bark. Systolic blood pressure (SBP) and heart rate were measured weekly using the pneumatic tail-cuff method (LETICA Scientific Instruments, BIOSEB). Vascular reactivity studies were performed 24 h after the last dose of treatment for each group of rats.

The dose of CG corresponded to 16 times the concentration that produced maximal relaxation of rat aortic rings in vitro (24). Because only 1–5% of natural molecules such as polyphenols and coumarins (25, 26) could be absorbed in the digestive tract, it can be assumed that the concentration of products present in the plasma was comparable to the concentration at which CG produced endothelium-dependent relaxation in rat aortic rings.

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Arterial preparation and mounting. The thoracic aorta and segments of branch II of SMAs (100–200 μm) were removed and carefully cleaned of adhering fat and connective tissue. They were mounted on a myograph filled with physiological salt solution (PSS) under normalized tension as previously described (17). PSS was maintained at 37°C and continuously bubbled with a 95% O₂-5% CO₂ mixture. In some experiments, the endothelial layer was removed by gentle rubbing of the intima with curved forceps for the aorta or a metallic wire for SMAs.

The presence of functional endothelium was assessed in all preparations by determining the ability of ACh (1 μM) to induce >50% relaxation of rings precontracted with a maximally active concentration of NE (1 μM for the aorta and 10 μM for the SMA, respectively). The absence of relaxation to ACh indicated that the vessels were functionally denuded of their endothelium.

Concentration-response curves to NE were constructed in a cumulative manner in arteries with or without functional endothelium. To characterize the involvement of NO and cyclooxygenase (COX) products, some arteries were exposed to the NOS inhibitors l-N^0-nitro-arginine methyl ester (l-NAME; 300 μM for the aorta) and N^0-nitro-arginine (l-NNA; 30 μM for the SMA) and the COX inhibitor indomethacin (10 μM). All the inhibitors were used at maximally active concentrations and were incubated with the tissue for 30 min before the concentration-response curves of NE were constructed. Only one concentration-response curve was constructed on each artery in either the absence or presence of the indicated inhibitor(s) for both the aorta and SMA.

Relaxation experiments. Endothelium-dependent relaxation was studied in aortic rings and SMAs with functional endothelium and precontracted to 80% of the maximal response obtained with NE (0.3 μM for the aorta and 3 μM for the SMA, respectively). This level of precontraction was chosen to better control the contraction in either the absence or presence of pharmacological inhibitors. The concentration-response curve for ACh was constructed on each artery with functional endothelium. In the aorta, the involvement of ROS in the relaxation to ACh was studied with the use of a combination of SOD (100 IU) plus catalase (1,000 IU). All the inhibitors were incubated with the tissue for 25 min before the precontraction with NE. Only one concentration-response curve was constructed on each artery in either the absence or presence of the indicated inhibitor(s) for the aorta.

Western blotting. Aortic rings were homogenized in lysis buffer, and 1 or 75 μg of total proteins (for SOD and NOS, respectively) from the supernatant fraction were loaded onto 15% or 7% SDS-PAGE (for SOD and NOS, respectively) from 2.55 g of total proteins (for SOD and NOS, respectively) from a n and or 75 μg of total proteins (for SOD and NOS, respectively) from 2.55 g of total proteins (for SOD and NOS, respectively). Proteins were transferred to nitrocellulose membranes and probed with rabbit anti-Cu/Zn SOD polyclonal antibody (Stressgen Biotechnologies by TEBUBio; Le Perray en Yverdon, Switzerland) or monoclonal mouse anti-eNOS and anti-iNOS (BD Biosciences; Le Pont de Claire, France). The bands were visualized using the enhanced chemiluminescence system (ECL Plus, Amersham; Buckinghamshire, UK) and quantified by densitometry.

Expression of results and statistical analysis. SBP in vivo was expressed in millimeters of mercury and heart rate was expressed in beats per minute. Vascular contraction was expressed in grams for the aorta and in milliNewton per millimeter for the SMA. The level of precontraction with NE was not significantly different within the two types of arteries (2.2 ± 0.1 and 2.1 ± 0.1 g, n = 16, for the aorta and 2.55 ± 0.36 and 3.09 ± 0.39 mN/mm, n = 6, for the SMA of control and CG-treated groups, respectively). Therefore, the relaxations were expressed as a percentage of the decrease in the level of precontraction. All results are expressed as means ± SE. Sensitivities to agonists were expressed as the maximal effect (E_{max}) and pD₂, where pD₂ = −log EC_{50} (where EC_{50} is the effective concentration that induced 50% of E_{max}). For Western blot analysis, results were expressed as a percentage of staining compared with the control, which was taken as 100%. Two-way ANOVA was used to compare concentration-response curves to agonists. Differences between means were assessed with Student’s t-test for unpaired data. Differences were considered significant when P < 0.05.

Drugs and chemicals. Dried and powdered trunk bark of CG (1 kg), collected in the northwest region of Madagascar in August 1999 (Voucher Specimen No. 4118-SF), was exhaustively extracted with 80% aqueous ethanol by maceration at room temperature to give a dark viscous extract. During the treatment, the final concentration of alcohol administered to CG-treated rats was 4%. Control rats were treated with the vehicle (5% glucose), which also contained the same proportion of alcohol. All the chemicals were purchased from Sigma Chemical (Grenoble, France).

RESULTS

SBP and heart rate in vivo. The SBP and heart rate of rats who received the vehicle and CG were not significantly different before the beginning of the treatment. Treatment of rats during 4 wk with the vehicle did not induce any change in SBP, although oral administration of 80 mg·kg⁻¹·day⁻¹ CG by gavage induced a progressive decrease of SBP (Fig. 1A). In contrast, heart rates of rats who received vehicle or CG were not significantly different during treatment either within the groups or between the groups (Fig. 1B).

Contraction response to NE. NE induced contraction in a concentration-dependent manner in aortic rings with and with-
without endothelium (Fig. 2). In aortic rings with functional endothelium, the COX inhibitor indomethacin (10 µM) did not significantly modify the response to NE in arteries taken from either control or CG-treated rats, even though the response was slightly reduced in the latter compared with the former (Fig. 2, A and B). In aortic rings without functional endothelium, the presence of the NOS inhibitor L-NAME (300 µM) did not affect contractile responses to NE in control groups (Fig. 2C). However, L-NAME significantly potentiated the response to the same agonist in aortas taken from the CG-treated group (Fig. 2D). It should be noted the concentration-response curve to NE was significantly depressed in aortas from the CG-treated group compared with control in the absence of functional endothelium (Fig. 2C and D). The hyporeactivity observed in aortas from the CG-treated group was completely restored toward control in the presence of L-NAME.

In SMAs, NE also induced concentration-dependent contraction, and this response was not significantly different in SMAs taken from control and CG-treated groups (Fig. 2, E and F, and Table 1). In the presence of functional endothelium, blockade of NO synthesis with L-NNA (30 µM) did not significantly affect contraction to NE in SMAs from control rats (Fig. 2E), but it potentiated the same response in SMAs from CG-treated rats (Fig. 2F). These results suggest an increased participation of endothelial NO in the regulation of contractility in SMAs from CG-treated group. However, there was no difference in concentration-response curves to NE in SMAs without endothelium in the absence or presence of NO synthase inhibitor L-NNA.

Table 1. pD2 values and E_{max} in response to norepinephrine in small mesenteric arteries

<table>
<thead>
<tr>
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<th>pD2 Values</th>
<th>E_{max} mN/mm</th>
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<tr>
<td></td>
<td>Control</td>
<td>CG</td>
</tr>
<tr>
<td>+E</td>
<td></td>
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<tr>
<td>+E + indomethacin</td>
<td>6.05±0.03</td>
<td>5.69±0.14</td>
</tr>
<tr>
<td>+E + L-NAME</td>
<td>6.27±0.05</td>
<td>5.92±0.12</td>
</tr>
<tr>
<td>-E</td>
<td>6.12±0.13</td>
<td>6.45±0.09</td>
</tr>
<tr>
<td>-E + indomethacin</td>
<td>6.96±0.16</td>
<td>6.44±0.05</td>
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Values are means ± SE; n = 4–6 experiments. Shown are pD2 values and maximal effect (E_{max}) in response to norepinephrine in small mesenteric arteries with (+E) or without endothelium (−E) in the presence or absence of the cyclooxygenase inhibitor indomethacin or the nitric oxide synthase inhibitor L-NNA (L-NNA). CG, Cedrelopsis grevei. *P < 0.05, statistically significant vs. CG + E.
l-NNA. There was no difference in concentration-response curves to NE in SMAs either with or without functional endothelium and in the absence or presence of COX (indomethacin, 10 μM). The pD2 values and E<sub>max</sub> of NE in all of those cited conditions are presented in Table 1.

**Endothelium-dependent relaxation.** ACh produced relaxation in a concentration-dependent manner in both the aorta and SMA with functional endothelium precontracted with NE (Fig. 3). ACh-induced relaxation was significantly potentiated in the aorta (Fig. 3A) and SMA (Fig. 3B) taken from the CG-treated group compared with those from the control group. The presence of superoxide anion and hydrogen peroxide scavengers [i.e., SOD (100 IU) and catalase (1,000 IU), respectively] did not affect the relaxation response to ACh in aortic rings from the control group (Fig. 4). However, the increase in relaxation observed in the aorta from the CG-treated group was prevented by the presence SOD plus catalase such that the response to ACh was not significantly different from that of the aorta taken from control rats.

**Western blot analysis.** The 140-kDa eNOS isoform and 130-kDa iNOS isoform were expressed in aortas from the control and CG-treated groups (Fig. 5). However, CG treatment enhanced eNOS but not iNOS expression in the aorta. The use of anti-Cu/Zn SOD helped to detect the presence of a 19-kDa protein in both groups, and this expression was significantly increased in aortas from CG-treated rats.

**DISCUSSION**

This study provides evidence that oral treatment with 80 mg·kg<sup>-1</sup>·day<sup>-1</sup> of extract of CG trunk bark during 4 wk induces a decrease in SBP without any alteration of heart rate. Also, CG treatment maintained the response to vasoconstrictor agent by a subtle adaptive mechanism involving nonendothelial NO and endothelial vasoconstrictor products, the nature of which remains to be determined. Most importantly, CG treatment improved endothelial function in both conductance and...
resistance arteries. The mechanism involved an increase of eNOS, Cu/Zn SOD protein levels, and a SOD plus catalase-sensitive pathway.

In previous work (24), we reported that CG was able to produce ex vivo relaxation in rat aortic rings and that its potency was greater in the presence of functional endothelium. The present study provides evidence that in vivo administration of CG induced a progressive decrease in blood pressure in the rat without any alteration in heart rate. The molecular identity of the compounds responsible for the in vivo effect described in the present study has not been assessed. However, administration of 80 mg·kg⁻¹·day⁻¹ CG for 4 wk is adequate to produce a sufficient circulating concentration of compounds to induce cardiovascular effects.

In accordance with data reported in the literature (21, 27), fractionation studies of CG lead to the isolation of coumarins, triterpenes, chromones, and polyphenols (O. Rakotoarison; unpublished data). Five coumarins have been isolated: norbraylin, methyl-Ö-cedrelospin, cedrecoumarin A, scoparone, and braylin, and they are among the constituents responsible for the vasorelaxing activity observed for the crude extract (24). These types of molecules are already known to be active on in vivo hemodynamic parameters (12, 31). In the case of polyphenols derived from red wine, this action on hemodynamic parameters involves the NO pathway (3). With regard to CG, it is reported that this plant extract induced an endothelium-dependent and -independent relaxation of the rat thoracic aorta. The endothelium-dependent component of the response was completely prevented by NOS and guanylyl cyclase inhibitor, suggesting the involvement of the NO pathway (O. Rakotoarison; unpublished observations). It should be noted that some of chemical constituents of CG have been described as antihypertensive and vasodilator (for scoparone (24, 28)) or as antioxidant (21). In addition, CG contains flavonoids like quercertin, which is known to have many beneficial effects as an antihypertensive and antioxidant (12). Those properties might participate in the cardiovascular effect of CG reported in the present study. However, further studies are needed to sort out the underlying mechanism.

Turning now to the effect of CG treatment on vascular reactivity in response to a vasoconstrictor agonist, we note that the contractions to NE were unchanged by CG in the aorta with functional endothelium. In endothelium-denuded aortic rings from CG-treated rats, the NE-induced contraction was decreased, and this hyporeactivity was prevented by the NOS inhibitor l-NAME. These data are consistent with the hypothesis that CG treatment elicits an extraendothelial production of NO, which leads to vascular hyporeactivity toward NE. Increased NO availability might result either from an increase of its production or a decrease of its breakdown. The production of NO did not originate from eNOS, although eNOS expression was enhanced in intact arteries because the response was obtained in the absence of functional endothelium. We detected a low level of iNOS expression by Western blot that was not different between control and CG-treated rats. This result suggests that at least a change in iNOS expression is not implicated in the hyporeactivity to vasoconstrictor agonist in the aorta from CG-treated rats without functional endothelium but a change in iNOS activity cannot be excluded. However, the reversal of vascular hyporeactivity in the presence of l-NAME in the endothelial-denuded preparation is in favour of nonendothelial NO. Whether this is due to a change in iNOS activity or an involvement of neuronal NOS, as reported by Boulanger et al. (6), is unknown; we cannot distinguish between these possibilities. Nevertheless, these data imply that there is a subtle mechanism that counterbalances the effect of extraendothelial NO production in intact aortas taken from CG-treated rats that allows the maintenance of NE-induced contraction. Several hypotheses could be advanced to explain such an effect. It could result from the participation an endothelium-derived vasoconstrictor factor from COX, as reported recently by Diebolt et al. (11). The COX inhibitor indomethacin did not significantly decrease the contraction to NE in CG-treated aortas with functional endothelium. CG treatment might increase the production of endothelial vasodilator products from COX that masked the effect of vasoconstrictor eicosanoids and therefore did not allow a significant reduction of the response in the presence of indomethacin. Alternatively, the production of endothelial eicosanoids after CG treatment might interfere with the activity of iNOS. Indeed, a complex interaction between iNOS and COX in regulating vascular reactivity has been reported such that an increased of COX products may lessen the vasodilatory effect of iNOS-derived NO under specific conditions (29).

The contractile response to NE in SMAs was not modified by treatment with CG in either the presence or absence of endothelium. In contrast to the aorta, blockade of NO synthesis revealed an enhanced participation of endothelium but not extraendothelial NO in the NE-induced contraction in SMAs from CG-treated rats. These data highlight vascular heterogeneity in response to CG treatment in the cellular source of NO involved in the regulation of vascular contraction.

The most important finding of this work relates to the effect of CG treatment on vascular relaxation. CG treatment increased the endothelial vasodilatation response to ACh in both the aorta and SMA. The nature of endothelial relaxant factors released in the two types of arteries is different. For the resistance arteries, many relaxant factors, such as NO, prostacyclin, and EDHF are involved in the relaxation response to ACh (18). The mechanisms involved in the increased relaxation observed in SMAs have not been investigated. For the aorta, the major endothelial factor released in response to ACh is NO. The increased potency of ACh-induced relaxation of the aorta after CG treatment may be due to either an increase in NO release or a protection of its breakdown. ACh-induced relaxation in arteries from both control and CG-treated rats implicates the NO pathway, in as much as it was completely prevented by the NOS inhibitor (data not shown). The increased ACh-induced relaxation can partially be attributed to an increased expression of eNOS protein even though an increased eNOS activity cannot be ruled out. It should be noted that, although the relaxation response to ACh was increased, the endothelial-dependent vasorelaxations to the sarco(endo)plasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin and to the Ca²⁺ ionophore A-23187 were not modified by CG treatment (results not shown). In contrast to thapsigargin and A-23187, which increase the cytosolic calcium concentration ([Ca²⁺]_), by direct actions on Ca²⁺ handling, ACh acts via second messengers. It can therefore be hypothesized that CG treatment improves endothelial-dependent vasodilatation by amplifying the pathway leading to second messenger production, such as the activity of phospholipase C. Enhanced expression eNOS expression upon
CG treatment is probably involved but was not sufficient to observe an increase of endothelium-dependent dilation in response to agents that act only via the modulation of Ca\(^{2+}\) handling. Thus the combination of both enhanced expression of eNOS and modulation of its activation through the Ca\(^{2+}\) pathway and second messenger production acting via phosphorylation is needed. The latter may explain the differential effect observed among the responses to ACh, thapsigargin, and A-23187. It is also interesting to note that the potentiating effect of CG on ACh-induced relaxation is attenuated in the presence of the superoxide anion and hydrogen peroxide scavengers, SOD plus catalase, suggesting an involvement of ROS. Similar mechanisms have been reported in the aorta of the rat treated with polyphenols from red wine (11). Superoxide anions have a complex interaction with both the intracellular pathway leading to NO production and NO itself. Superoxide anions could react with NO and then decrease NO bioavailability. On the other hand, superoxide anions might be involved in the transduction mechanism leading to an increase in [Ca\(^{2+}\)]\(_i\) within the endothelial cells upon CG treatment. Furthermore, the expression of the antioxidant enzyme Cu/Zn SOD was also enhanced in the aorta after CG treatment. The latter might be implicated in the enhanced endothelial NO vasodilatation resulting from an increased H\(_2\)O\(_2\) production from superoxide anions known both to possess vasodilatory properties through a mechanism sensitive to catalase (7) and to enhance agonist-stimulated Ca\(^{2+}\) signaling and the phosphorylation pathway leading to NO production in endothelial cells (8, 30). Altogether, this complex interaction between NO and ROS might explain why the enhancement of NO endothelial vasodilation upon CG treatment through ROS scavenger-sensitive mechanisms could be observed with an agent that can activate both an increase in Ca\(^{2+}\) and a phosphorylation cascade only.

The present study shows that oral administration of CG produces a decrease in blood pressure in normotensive rats. This hemodynamic effect was associated with increased endothelial NO-mediated relaxation through a mechanism sensitive to SOD plus catalase and with an increase of eNOS and Cu/Zn SOD protein expression in the vessel wall. Vascular contractility in the aorta was maintained unchanged possibly due to enhanced extraendothelial NO vasodilatation compensated for by unknown endothelial constrictor products. The present study may contribute to a better understanding of the cardiovascular properties of CG. This information is of importance for populations who use this plant in traditional medicine and encourage us to continue the pharmacological study of this medicinal plant, with the aim of finding new drugs useful for the treatment of cardiovascular diseases.

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