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Dioclein, a new nitric oxide- and endothelium-dependent vasodilator flavonoid

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Abstract

In the present work, the vasorelaxant effect of dioclein, a new flavonoid isolated from *Dioclea grandiflora* (Leguminoseae), was investigated in the rat aorta. Dioclein induced a concentration-dependent relaxation in vessels pre-contracted with phenylephrine ($IC_{50} = 1.3 \pm 0.3$ μM), a response which was abolished after endothelium removal. Neither indomethacin (10 μM), an inhibitor of cyclo-oxygenase, nor atropine (1 μM), an antagonist of muscarinic receptors, modified the effect of dioclein. Dioclein (30 μM) induced a significant increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in aortic rings with endothelium. The nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine-methyl-ester (L-NAME, 300 μM), strongly inhibited or abolished the relaxing effect and rise in cyclic GMP levels induced by dioclein. Furthermore, dioclein (30 μM) had no effect on the endothelium-independent relaxation produced by the NO donor, 3-morpholino-sydnonimine (SIN-1), while superoxide dismutase (100 U ml^{-1}) significantly potentiated it. These results indicate that, in the rat aorta, dioclein induces a NO- and endothelium-dependent vasorelaxant effect, which is associated with cyclic GMP elevation. This vasorelaxation likely results from enhanced synthesis of NO rather than enhanced biological activity of NO.

Keywords: Flavonoid; Dioclein; Endothelium; Vasorelaxation; Nitric oxide (NO); Aorta

1. Introduction

There has been a great deal of interest in polyphenolic compounds due to their beneficial cardiovascular effects in humans (Stanley and Mazier, 1999). Consumption of flavonoids in the diet has been shown to be inversely associated with morbidity and mortality from coronary heart disease (Hertog et al., 1993, 1995; Muldoon and Kritchevsky, 1996; Knekt et al., 1996). Flavonoids have been widely described in the literature as vasodilator compounds (Duarte et al., 1993; Fitzpatrick et al., 1993; Herrera et al., 1996). Most of their effects are reported to be independent of the presence of an intact endothelium (Duarte et al., 1993; Herrera et al., 1996). However, recently, an endothelium-dependent vasodilator effect was described for some types of flavonoids (Fitzpatrick et al., 1993; Andriambeloson et al., 1997, 1998). Furthermore, flavonoids inhibit platelet aggregation (Seigneur et al., 1990; Fitzpatrick et al., 1993) and their antioxidant properties may delay the onset of atherogenesis by reducing peroxidative reactions and decreasing thrombotic tendency (Rice-Evans et al., 1997; Aviram and Fuhrman, 1998; Stanley and Mazier, 1999).

Some experimental data support a role for an enhancement by flavonoids of nitric oxide (NO) generation in endothelial cells (Andriambeloson et al., 1997, 1998). Endothelial NO plays a major role in the control of vasomotor tone and structure (Lüscher, 1994; Vanhoutte and Boulanger, 1995). In pathological conditions such as hypertension and atherosclerosis, there is a dysfunction in

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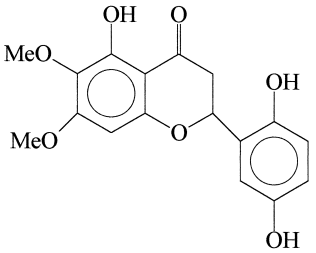

Fig. 1. Structure of dioclein (5, 2′, 5′-trihydroxy-6-7-dimethoxyflavanone).

the vascular endothelium with a subsequent reduction in the release, bioavailability or action of endothelium-derived relaxing factors (Lüscher, 1994). Thus, NO release or function has been shown to be decreased in hypertensive subjects and in animal models of hypertension (Lüscher, 1994; Vanhoutte and Boulanger, 1995). Furthermore, an altered release of NO also seems to be involved in the pathophysiology of atherosclerosis (Strijker Boudiêr et al., 1995). Therefore, the development of vasodilator compounds with the ability to restore the levels of NO in the vascular system could potentially contribute to the treatment of these cardiovascular diseases. However, it should be considered that in some pathological conditions the constitutive NO synthase seems to induce an increased production of superoxide ions due to an insufficiency of tetrahydrobiopterin (Cosentino et al., 1998).

In view of the potential beneficial effects of flavonoids in the cardiovascular system, the aim of the present study was to evaluate the potential vasodilator activity of the new flavonoid dioclein (Fig. 1), isolated from *Dioclea grandiflora* (Bhattacharyya et al., 1995). This plant is a vine that grows in the coastal plain of northeastern Brazil. An infusion of the roots of this plant is used for the treatment of kidney stones and prostate gland disorders, and the seeds are used in the human diet in the ''Caatinga'' regions of Brazil (Lima, 1989). The study was performed with the rat aorta, a well-known vascular preparation, and we report here for the first time the vasorelaxant effect of dioclein and its dependence on the release of the endothelium-derived NO in the rat aortic rings.

2. Materials and methods

2.1. Rat aortic rings preparation and mounting

Male Wistar rats (200–250 g) were killed by cervical dislocation and exsanguinated. The descending thoracic aorta was excised, free of fat and connective tissue, cut into rings about 4–5 mm in length and set up in gassed (95% O_2 and 5% CO_2) Krebs–Henseleit solution of the following composition (mM): NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, NaH_2PO_4 2.33 and glucose 11.51. When necessary, the endothelium was removed by rubbing the intimal surface with a wooden stick. The tissues were maintained at 37°C under a tension of 1 g and equilibrated for a period of 1 h before initiating experimental protocols. During this period, the incubation medium was changed every 15 min. After the equilibration period, two contractile responses were evoked by submaximal concentrations of phenylephrine (0.3 μM) to elicit reproducible responses. The presence of functional endothelium was assessed by the ability of acetylcholine (1 μM) to induce more than 50% relaxation of vessels pre-contracted with phenylephrine (0.3 μM). The absence of acetylcholine relaxant activity indicated the absence of a functional endothelium. The responses of the tissues were recorded using isometric transducers and physiographs (Ugo Basile).

2.2. Vasorelaxant activity in pre-contracted rat aortic rings

The vasorelaxant activity of dioclein was measured in aortic rings with or without functional endothelium pre-contracted to the same tension (approximately 1.5 g of tension) with submaximal concentrations of phenylephrine (0.3 or 0.1 μM, respectively). Dioclein was added in increasing cumulative concentrations once the response to phenylephrine had stabilized. In order to verify the participation of endothelium-derived products and of muscarinic receptors to the relaxant effect of dioclein, experiments were performed in the presence of L-NAME (N^G nitro-L-arginine-methyl-ester; 300 μM), indomethacin (10 μM) or atropine (1 μM), added to the bath 20 min prior to the addition of phenylephrine. In another set of experiments performed in endothelium-denuded preparations, dioclein (30 μM) or superoxide dismutase (100 U ml^{-1}) was added when the contractile response to phenylephrine (1 μM) reached a steady-state tension. Ten minutes later, concentration–response curves for 3-morpholino-sydnonimine (SIN-1) were made.

2.3. Measurement of cyclic GMP

Rat aortic rings with functional endothelium (3–4 mm in length) were incubated at 37°C in gassed (95% O_2 and 5% CO_2) Krebs–Henseleit solution containing isobutyl-methylxanthine (IBMX, 100 μM, in order to inhibit cyclic GMP degradation through cyclic nucleotide phosphodiesterases), superoxide dismutase (100 U ml^{-1}, to prevent NO degradation by O_2^-) and catalase (100 U ml^{-1}, to remove H_2O_2). Rings were exposed (for 5 min) either to 0.3% dimethyl sulfoxide (DMSO, the vehicle of dioclein) or to dioclein (30 μM, a concentration producing maximal endothelium-dependent relaxation), in the absence or in the presence of L-NAME (300 μM, added 10 min before the exposure to dioclein). The reaction was stopped by transferring the rings to 1 ml of an ice-cold $HClO_4$ solution (1.07 N). Following homogenization and centrifugation

(10.000 × *g* for 10 min), the supernatant was collected for cyclic GMP determination using radioimmunoassay (cyclic GMP IMMUNOTECH kit, Marseille, France). The DNA content of the pellet was measured as described by Brunk et al. (1979).

2.4. Drugs

Acetylcholine chloride, atropine sulfate, catalase, IBMX, indomethacin, L-NAME, L-phenylephrine chloride, SIN-1, and superoxide dismutase were purchased from Sigma. Indomethacin was dissolved in 0.5% w/v sodium bicarbonate. The isolation and identification of dioclein is described in detail elsewhere (Bhattacharyya et al., 1995). For contractile experiments, dioclein was solubilized in a mixture of distilled water/chremophor at a concentration of 10 mM and diluted to the desired concentration with distilled water just before use. The final concentration of chremophor in the bath never exceeded 0.01% and was without effect when tested in control preparations (data not shown). In the experiments for measurements of cyclic GMP, dioclein was dissolved as a 10-mM solution in DMSO and further dilutions were made in distilled water. The other compounds were freely dissolved in distilled water.

2.5. Data analysis

Results are expressed as means ± S.E.M. Results from contractile experiments are expressed as percentage decreases in the maximal contraction induced by phenylephrine, and the point when the basal line was reached was considered 100% relaxation. Values of inhibitory concentration 50% (IC_{50}) were calculated graphically from the individual concentration–response curves by non-linear curve fitting. Cyclic GMP content is expressed as picomole per microgram DNA. Student's *t*-test was used to analyse the data, and results were considered significant when $P < 0.05$.

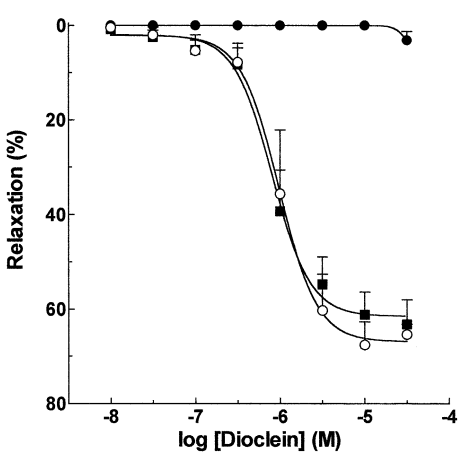

Fig. 2. Vasodilator effect of dioclein in rat aortic rings with (○) or without (●) functional endothelium. Experiments with rings with a functional endothelium were performed in the absence (○) or in the presence (■) of the muscarinic antagonist, atropine (1 μM). The values are means ± S.E.M. of at least five experiments.

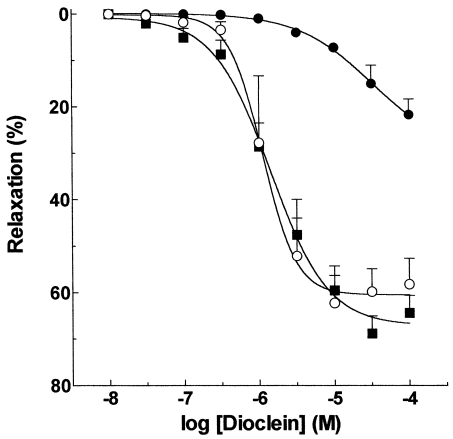

Fig. 3. Vasodilator effect of dioclein in rat aortic rings with a functional endothelium, in the absence (○) or in the presence of L-NAME (300 μM; ●) or indomethacin (10 μM; ■). The results are means ± S.E.M. of five experiments.

3. Results

3.1. Effect of dioclein on the sustained contraction induced by phenylephrine in endothelium intact and denuded aorta

In aortic rings with functional endothelium, dioclein (0.01 to 30 μM) induced a concentration-dependent relaxation of the sustained contractions induced by phenylephrine (Fig. 2). The IC_{50} value for dioclein was 1.3 ± 0.3 μM ($n = 6$). The basal tone of the rat aorta was not changed by concentrations of dioclein up to 30 μM (not shown). In rings without functional endothelium, dioclein in concentrations up to 30 μM did not produce any significant relaxant effect (Fig. 2). These results demon-

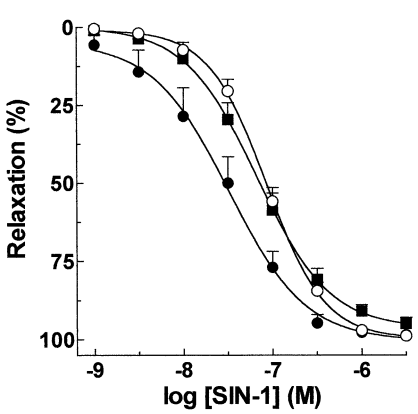

Fig. 4. Vasodilator effect of SIN-1 in rat aortic rings without a functional endothelium, in the absence (○) or in the presence of dioclein (30 μM; ■), or superoxide dismutase (100 U ml^{-1}; ●). The results are means ± S.E.M. of five experiments.

strate that, in the rat aorta, dioclein had a vasorelaxant effect dependent on the presence of a functional endothelium.

3.2. Mechanism involved in the endothelium-dependent vasorelaxation induced by dioclein

The selective antagonist of the muscarinic receptor, atropine, at a concentration (1 μM) at which it completely inhibited acetylcholine (0.001 to 1 μM)-induced relaxation (not shown), had no effect on the vasorelaxant effect of dioclein (Fig. 2). The IC_{50} value for dioclein in the presence of atropine was 1.1 ± 0.2 μM ($n = 5$). Blockade of cyclo-oxygenase by indomethacin (10 μM) did not affect the dioclein-induced relaxation (Fig. 3). The IC_{50} value for dioclein in the presence of indomethacin was 1.7 ± 0.6 μM ($n = 5$). However, the endothelium-dependent vasorelaxant effect of dioclein was strongly inhibited by L-NAME (300 μM), a selective inhibitor of NO synthase (Fig. 3).

3.3. Effect of dioclein on the relaxation produced by the NO donor, SIN-1 in endothelium-denuded preparations

In endothelium-denuded rings pre-contracted with phenylephrine (1 μM), SIN-1 (0.001–30 μM) produced a concentration-dependent relaxation (Fig. 4). Addition of superoxide dismutase produced a leftward shift in the concentration–response curve for SIN-1. The IC_{50} value of SIN-1 in the presence of superoxide dismutase (100 U ml^{-1}) was significantly decreased from 89 ± 14 to 33.4 ± 11 nM ($n = 5$, $P < 0.05$; Fig. 4). By contrast, dioclein (30 μM) at a concentration that produced maximal effects in endothelium-intact vessels did not modify the vasorelaxant effect of SIN-1 (Fig. 4). The IC_{50} value of SIN-1 in the presence of 30 μM dioclein was 72 ± 13 nM.

3.4. Effect of dioclein on cyclic GMP content in endothelium-intact aorta

Dioclein (30 μM) produced a significant increase in cyclic GMP content in the rat aortic rings with functional endothelium (Fig. 5). The increase in cyclic GMP content induced by dioclein was not observed in rings pre-treated with the NO synthase inhibitor L-NAME (300 μM; Fig. 5).

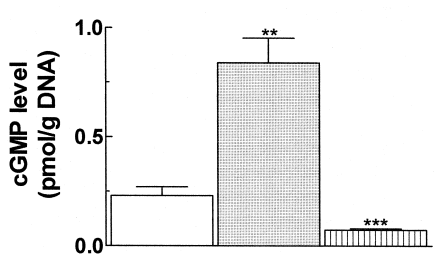

Fig. 5. Cyclic GMP content of rat aortic rings with a functional endothelium in the absence (empty square) or in the presence of dioclein (30 μM; dotted square) or dioclein (30 μM) plus L-NAME (300 μM; square with vertical line). The values are means ± S.E.M. of four experiments. $^{**}P < 0.01$ vs. control values and $^{***}P < 0.001$ vs. dioclein values.

4. Discussion

The present work was performed in order to investigate possible vasodilator effects of the new flavonoid dioclein in the rat aorta. It was observed that dioclein induced a NO- and endothelium-dependent vasorelaxation in rat aortic rings.

Several lines of evidence support the idea that the effect of dioclein was dependent on the production of NO from endothelial cells. The vasorelaxation induced by dioclein was dependent on the presence of functional endothelium and was reduced after inhibition of NO synthase activity by L-NAME. Moreover, dioclein at a concentration, which had no direct relaxant effect in endothelium-denuded vessels increased the cyclic GMP level in endothelium-intact rat aorta and this increase was abolished in the presence of L-NAME. Thus, it is unlikely that the direct activation of guanylyl cyclase or the inhibition of phosphodiesterases in smooth muscle accounted for the vasorelaxing effect of dioclein.

It is well known that NO induces vascular smooth muscle relaxation through activation of guanylyl cyclase, leading to the accumulation of cyclic GMP (Moncada et al., 1991). Therefore, an endothelium-dependent and L-NAME-sensitive vasorelaxation and cyclic GMP elevation can result from an increased production of NO or from a diminution of its breakdown. It is well established that NO interacts with O_2^- to produce peroxynitrite, resulting in a decreased vasodilating effect (Butler et al., 1995). Polyphenolic flavonoids are powerful antioxidants and exert free radical-scavenging properties (Robak and Gryglewski, 1988; Rice-Evans et al., 1997). In the present study, the possibility that the relaxant effect of dioclein resulted from prevention of NO breakdown through O_2^- removal was investigated. It is demonstrated that, in endothelium-denuded preparations, dioclein (at a concentration that produced maximal effects in endothelium intact vessels) did not modify the vasodilator effect of SIN-1, a drug that releases NO and O_2^- spontaneously (Feelisch et al., 1989). In contrast, superoxide dismutase, which removes extracellular O_2^-, potentiated the vasorelaxant effect of SIN-1 showing that, under our experimental conditions, scavenging of O_2^- was functional. Thus, our results did not support the idea that the vasorelaxation induced by dioclein was due to a protection of NO breakdown by extracellular O_2^-. This is also supported by the observation that, in the presence of functional endothelium, dioclein was able to produce cyclic GMP elevation in the presence of superoxide dismutase and catalase. Therefore, the relaxant effect of dioclein involved stimulation of the production of NO by endothelial cells.

The possibility that endothelial vasorelaxant factors derived from cyclo-oxygenase participated in the relaxant effect of dioclein is unlikely since indomethacin had no effect against dioclein. Similarly, stimulation of endothelial muscarinic receptors by dioclein probably did not account for dioclein-induced vasorelaxation, because atropine in concentrations, which completely blocked the endothelium-dependent relaxation induced by acetylcholine did not modify the dioclein-induced relaxation.

Vasodilator effects of flavonoids have already been reported in the literature. However, most of the flavonoids described to date exhibit a relaxant effect independent of the presence of a functional endothelium and are 5- to 300-fold less potent than dioclein (Duarte et al., 1993; Herrera et al., 1996). Very recently, an endothelium-dependent vasorelaxant effect was reported for some well-known flavonoids, namely, leucocyanidol and delphinidin (Amdriambeloson et al., 1997, 1998). The relaxant effect of these flavonoids was in the range of 20 μM, being approximately 10-fold less potent than dioclein. These observations support the relevance of the investigation of new compounds for the understanding of structure–activity properties involved in the endothelium-dependent vasorelaxing effect of flavonoids.

Endothelium-derived NO plays an important role in the control of vascular homeostasis. NO controls vascular tone, modulates the growth of vascular smooth muscle cells and decreases platelet adhesion and aggregation, as well as the adherence of other blood components (Moncada et al., 1991; Scott-Burden and Vanhoutte, 1993). A reduced production of NO by vascular endothelial cells is closely associated with the endothelial dysfunction or injury, which is proposed to be an important factor in severe pathologies such as atherosclerosis, restenosis and hypertension (Lüscher, 1994; Busse and Fleming, 1996). Therefore, the development of compounds with the ability to increase the production of NO by endothelial cells can be of great value for the treatment of these cardiovascular diseases. It is attractive to speculate that the beneficial effects of flavonoid consumption on cardiovascular morbidity and mortality may be related to their ability to enhance NO levels in the vasculature. Thus, dioclein could be used as potential lead compound for the development of drugs for the treatment of hypertension and atherosclerosis. However, in some pathological conditions where the cofactor tetrahydrobiopterin is deficient, the activation of constitutive NO synthase induces an increased production of superoxide (Consentino et al., 1998) and the effect of this flavonoid should also be evaluated in these pathological conditions.

In conclusion, the flavonoid dioclein produced a potent endothelium-dependent relaxation of the rat aorta, which probably resulted from an increase in NO production by endothelial cells. Further work is necessary to clarify the underlying mechanism involved in dioclein-induced increases in NO production.

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