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Inhibitory Effects of Daunorubicin on Endothelium-dependent Vasorelaxing Response to Acetylcholine of Rat Aorta

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Abstract—The effect of daunorubicin on the endothelium-dependent vasorelaxing response to acetylcholine was investigated using rat isolated aorta and compared with the effect of aclarubicin. Treatment of aortic strips with daunorubicin (20 μM) significantly attenuated the relaxing response to acetylcholine in the absence of tetraethylammonium, but not in its presence. Pretreatment with daunorubicin at a higher concentration (50 μM) or with aclarubicin (20 μM) strongly attenuated the relaxing response to acetylcholine; this attenuation was unaffected by the presence of tetraethylammonium. The increase in aortic cGMP in response to acetylcholine was also significantly suppressed by pretreatment with 50 μM daunorubicin or 20 μM aclarubicin, but not by treatment with 20 μM daunorubicin. The inhibitory effect of 20 μM aclarubicin on the acetylcholine-induced responses was stronger than that of 50 μM daunorubicin. Even in strips pretreated with both 50 μM daunorubicin and 20 μM aclarubicin, relaxation induced by 0·1 μM sodium nitroprusside was retained. These results suggest that daunorubicin at 20 μM inhibits the endothelium-dependent vasorelaxing response to acetylcholine via a mechanism other than the nitric oxide-mediated pathway, whilst at 50 μM, it inhibits the nitric oxide-mediated vasorelaxation.

Daunorubicin and aclarubicin are anthracycline antibiotics used in therapy against neoplastic disease. In addition to cardiotoxicity, they have recently been reported to affect vascular smooth muscle function in-vitro. In rat aorta, daunorubicin at 17·7 μM potentiates vasocontraction by facilitating activation of the voltage-dependent calcium channel (Wakabayashi et al 1990), and at 35·5–142 μM, daunorubicin alone can produce contractions dependent on both intra- and extracellular calcium (Wakabayashi et al 1989a). On the other hand, aclarubicin at 23·6–94·3 μM inhibits contraction of the rat aorta (Wakabayashi et al 1989b). Thus, daunorubicin and aclarubicin possess opposite actions on vascular smooth muscle contractility.

Vascular tone is regulated by substances from the endothelium (Furchgott & Vanhoutte 1989). Recently, aclarubicin at 5·9–23·6 μM in-vitro, has been shown to strongly inhibit endothelium-dependent vasorelaxation of the rat aorta (Wakabayashi et al 1991). Also, aortae from rats made nephrotic with daunorubicin injection have been found to display attenuation of endothelium-dependent relaxation (Ito et al 1991). However, the effect of daunorubicin in-vitro on endothelium-dependent vasorelaxation is not known.

The aim of this study was to investigate whether daunorubicin in-vitro affects the endothelium-dependent vasorelaxation by acetylcholine and to compare it with the inhibitory action of aclarubicin.

Materials and Methods

Measurement of isometric tension

Male Wistar rats, 350–450 g, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (50 mg kg^{-1}) and killed by exsanguination. The thoracic aortae were rapidly excised and placed in Krebs–Ringer bicarbonate solution (mM): NaCl 118, KCl 4·7, $CaCl_2$ 2·5, KH_2PO_4 1·2, $MgSO_4$ 1·2, glucose 10 and $NaHCO_3$ 25. After the removal of excess fat and connective tissue, helical strips (approx. 2 × 15 mm) were prepared for tension studies and ring strips (5 mm long) for measurement of cyclic (c) GMP.

Measurement of isometric tension

Each helical strip was suspended vertically in an organ bath containing 10 mL of the above solution maintained at 37°C and bubbled with 95% O_2–5% CO_2 (pH 7·4). The upper end of the preparation was connected to a force-displacement transducer (Nippon Kohden Kogyo Co., Tokyo, Japan). After 1-h equilibrium at a resting tension of 1 g, changes in the isometric force were recorded. During the equilibrium period the medium in the organ bath was replaced with fresh medium every 20 min. Relaxants were added when the phenylephrine-induced contraction reached a plateau. Only one concentration-response curve of the relaxing response to acetylcholine was obtained per preparation. The relaxant response was expressed in terms of the percentage decrease of tension/the contractile force before addition of the relaxant. Since incubation with 50 μM daunorubicin for 15 min produced a slight and gradual contraction, the contractile force before the addition of the relaxant was calculated as the force from the basal tension before drug addition to the peak tension of phenylephrine contraction. Tetraethylammonium (TEA) was given 20 min before the addition of phenylephrine. Daunorubicin or aclarubicin was added 15 min before the addition of phenylephrine. Since phenylephrine contraction was potentiated by TEA pretreatment, a smaller concentration (0·03 μM) of phenylephrine was used in the presence of TEA.

Measurement of tissue cGMP

Aortic-ring strips were preincubated for 1 h in tubes containing 5 mL Krebs–Ringer bicarbonate solution maintained at 37°C and bubbled with 95% O_2–5% CO_2 (pH 7·4).

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After drug exposure, the tissues were frozen in liquid nitrogen, then homogenized in 1 mL 6% trichloroacetic acid and centrifuged at 3000 *g* at 4°C for 10 min. The supernatants were extracted 4 times in 5 vol water-saturated ether, and the samples were evaporated to dryness. They were stored at −40°C until assayed for cGMP. After reconstitution of the samples in sodium acetate buffer (50 mM, pH 6·2) containing theophylline (1 mM) for acetylation, cGMP was determined by radioimmunoassay with a New England Nuclear kit (Boston, MA, USA). The tissue residue was solubilized in 2 M NaOH and neutralized with concentrated HCl, and protein was determined with a Bio-Rad dye-binding protein assay kit (Richmond, CA, USA) using bovine albumin as standard. The cGMP content of each strip was expressed as pmol (mg protein)$^{-1}$.

Drugs

The pharmacological agents used were phenylephrine hydrochloride, tetraethylammonium (Sigma Chemical Co. Ltd, St Louis, MO, USA), acetylcholine hydrochloride, sodium nitroprusside, theophylline (Wako Pure Chemical Co. Ltd, Osaka, Japan), nitro-L-arginine (Aldrich Chemical Co. Milwaukee, WI, USA), aclarubicin hydrochloride (Yamanouchi Seiyaku Co. Ltd, Tokyo, Japan), daunorubicin hydrochloride (Meiji Seika Co. Ltd, Tokyo, Japan) and sodium pentobarbitone (Abbott Laboratories, North Chicago, IL, USA). Aclarubicin and daunorubicin were dissolved in physiological saline to make up stock solutions of 2·36 and 3·55 mM, respectively, and kept at 4°C. The concentration of each drug was expressed as the final concentration in the organ bath.

Statistical analysis

The data were expressed as means ± s.e. Significance was determined using the Mann-Whitney U-test for percent relaxation data or Student's *t*-test for data on the cGMP levels, and *P* values less than 0·05 were considered significant.

Results

Effects of daunorubicin and aclarubicin on phenylephrine-induced contractile force

The increase of the isometric tension from the basal level to the peak tension induced by 0·5 μM phenylephrine (mg (mg tissue)$^{-1}$) was not significantly altered by daunorubicin (20 or 50 μM) pretreatment when compared with the control pretreated with saline (control, 186·6 ± 12·3; 20 μM daunorubicin, 189·8 ± 10·9; 50 μM daunorubicin, 193·2 ± 13·3). Treatment of the aortic strip with 20 μM aclarubicin slightly reduced the mean contractile force by 0·5 μM phenylephrine (161·2 ± 5·5 mg (mg tissue)$^{-1}$) compared with the control, but the difference was not significant.

Effects of daunorubicin and aclarubicin on acetylcholine-induced relaxation

Daunorubicin (20 or 50 μM) or aclarubicin (20 μM) pretreatment significantly attenuated the relaxing response to acetylcholine (Fig. 1A). When the acetylcholine-induced relaxing response had reached a plateau, addition of daunorubicin (50 μM) rapidly reversed the isometric tension and further addition of aclarubicin (20 μM) brought it above the phenylephrine-precontracted level (Fig. 2A). When the attenuated relaxing response to acetylcholine had reached a plateau in strips pretreated with daunorubicin (50 μM), addition of aclarubicin (20 μM) rapidly reversed the isometric tension to above the phenylephrine-precontracted level, and the subsequent addition of sodium nitroprusside (0·1 μM) relaxed the aortic strips to almost the basal tension (Fig. 2B). When the sodium nitroprusside-induced relaxing response had reached a plateau in the strips without daunorubicin or aclarubicin pretreatment, addition of daunorubicin (50 μM) could not reverse the isometric tension (Fig. 2C).

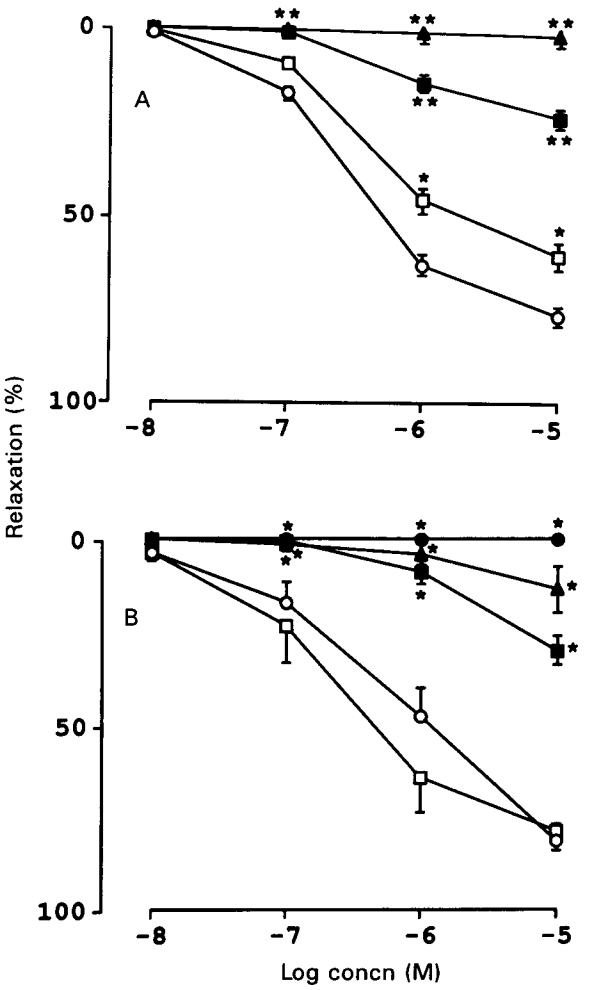

FIG. 1. Effects of daunorubicin and aclarubicin on relaxing response to acetylcholine in the absence (A) or presence (B) of TEA. A. Rat aortic strips were pretreated with daunorubicin, aclarubicin or vehicle (saline) for 15 min, and then contracted with 0·5 μM phenylephrine. After the phenylephrine contraction had reached a plateau, a relaxing response to acetylcholine occurred in a cumulative manner. B. Rat aortic strips were pretreated with 10 mM TEA for 20 min and with daunorubicin, aclarubicin, nitro-L-arginine or vehicle (saline) for 15 min, and then contracted with 0·03 μM phenylephrine. After the phenylephrine contraction had reached a plateau, a relaxing response to acetylcholine occurred in a cumulative manner. ○ Control, □ 20 μM daunorubicin, ■ 50 μM daunorubicin, ▲ 20 μM aclarubicin, ● 100 μM nitro-L-arginine. $*P<0·05$, $**P<0·01$ (A); $*P<0·01$ (B) compared with control.

Effects of daunorubicin and aclarubicin on acetylcholine-induced relaxation in the presence of TEA

The mean size of the contractile response to phenylephrine

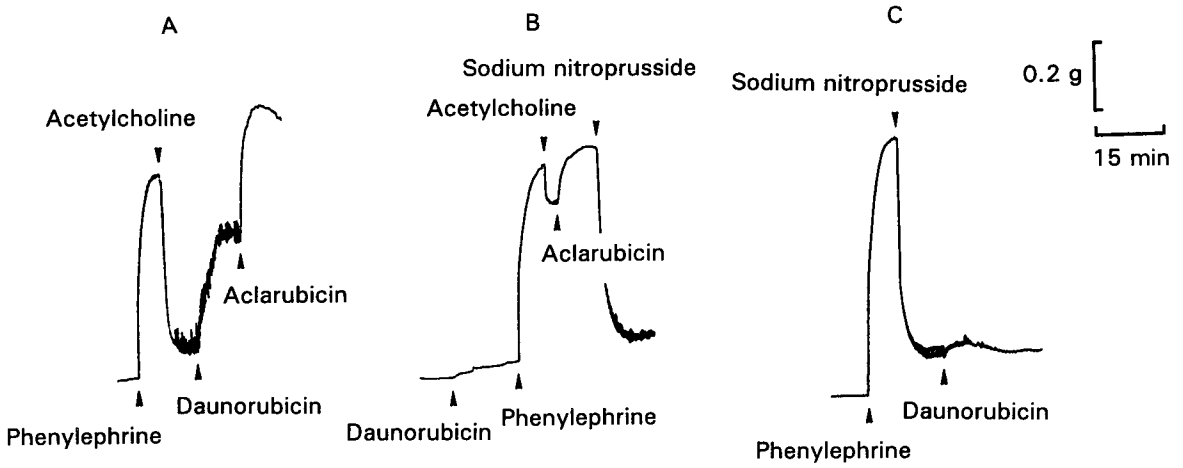

FIG. 2. Representative tension recording of rat aortic strips. Concentrations of drugs were: acetylcholine, 10 μM; sodium nitroprusside, 0·1 μM; aclarubicin, 20 μM; phenylephrine, 0·5 μM; daunorubicin, 50 μM.

Table 1. Effects of daunorubicin and aclarubicin on tissue cGMP of rat aortic rings. Rat aortic strips pretreated with daunorubicin, aclarubicin or vehicle (saline) for 15 min were contracted with 0·5 μM phenylephrine for 10 min, and then stimulated with 10 μM acetylcholine for 1 min.

Conditions	Tissue cGMP (pmol (mg protein)$^{-1}$)
Basal (without acetylcholine stimulation)	1·5 ± 0·1
Acetylcholine (10 μM)	28·6 ± 4·2*
Acetylcholine (10 μM) + daunorubicin (20 μM)	25·1 ± 3·0
Acetylcholine (10 μM) + daunorubicin (50 μM)	9·4 ± 1·5†
Acetylcholine (10 μM) + aclarubicin (20 μM)	2·5 ± 0·5†

Data are the mean ± s.e. of nine observations. *$P < 0·01$ compared with basal value, †$P < 0·01$ compared with acetylcholine alone.

(0·03 μM) in the presence of TEA was 162·0 ± 12·4 mg (mg tissue)$^{-1}$ (n = 8). Acetylcholine-induced relaxation in the presence of TEA was markedly attenuated by pretreatment with daunorubicin at 50 μM or aclarubicin at 20 μM, but not significantly altered by daunorubicin at 20 μM. Nitro-L-arginine (100 μM) completely blocked the relaxing response to acetylcholine in the presence of TEA (Fig. 1B).

Effects of daunorubicin and aclarubicin on cGMP in aortic tissue

Stimulation with acetylcholine greatly increased tissue cGMP. This increase could be significantly suppressed by pretreatment of the aorta with 50 μM daunorubicin or 20 μM aclarubicin, but not with 20 μM daunorubicin (Table 1).

Discussion

The endothelium-dependent vasorelaxation by acetylcholine is attributed to the release of endothelium-derived relaxing factor (EDRF) and endothelium-derived hyperpolarizing factor (EDHF). EDRF is now generally acknowledged to be nitric oxide (NO) or a nitrosoderivative releasing nitric oxide; it stimulates soluble guanylate cyclase, followed by an increase in cGMP (Ignarro et al 1987; Palmer et al 1987; Myers et al 1990). EDHF, a still unidentified factor, opens the K^+ channel and induces membrane hyperpolarization (Chen et al 1988; Taylor & Weston 1988).

A recent report by Ito et al (1991) showed that endothelium-dependent relaxation was significantly attenuated in aorta isolated from rats injected with daunorubicin. It is reasonable to assume that the impairment was due to the development of nephrosis, as this is accompanied by hypercholesterolaemia and hypertension, which are pathological conditions that attenuate endothelium-dependent vasorelaxation (Winquist et al 1984; Verbeuren et al 1986). However, the direct effects of daunorubicin in-vitro on endothelium-dependent vasorelaxation have not been reported.

TEA, a non-selective potassium-channel blocker inhibits the relaxing response mediated by EDHF in rat aorta (Bray & Quast 1991). In this study, the acetylcholine-induced relaxing response was completely inhibited in the presence of both TEA and nitro-L-arginine, a stereospecific inhibitor of NO production from L-arginine (Palacios et al 1989; Moore et al 1990). Thus, the relaxation response by acetylcholine in the presence of TEA was mediated only by NO from the vascular endothelium. Treatment of rat aortic strips with daunorubicin at 20 μM significantly attenuated endothelium-dependent relaxation by acetylcholine in the absence of TEA, but not in its presence. Furthermore, 20 μM daunorubicin did not significantly affect the increase in tissue cGMP in response to acetylcholine. Therefore, although we could not separately evaluate the effect of daunorubicin on the EDHF-mediated response, 20 μM daunorubicin does not affect the NO-mediated relaxing response to acetylcholine, but probably depresses the EDHF-mediated response. It is easy to understand that EDHF-mediated relaxation by acetylcholine in rat aorta is inhibited by daunorubicin, since daunorubicin facilitates activation of the voltage-dependent calcium channel in rat aorta, probably by membrane depolarization (Wakabayashi et al 1990).

In this study, daunorubicin at concentrations of 20 and 50 μM did not potentiate the contractile response to phenylephrine, in agreement with the previous finding that daunorubicin (17·7 μM) potentiates contractile responses to KCl and Bay K 8644, but not those to phenylephrine and clonidine (Wakabayashi et al 1990). Thus, daunorubicin selectively potentiates contraction by activation of the voltage-dependent calcium channel. Relaxant agonists do not relax vascular strips contracted to different tensions to equivalent percentages (Cohen & Berkowitz 1974). In the present study, contractile tension induced by 0·03 μM phenylephrine in the presence of TEA was slightly lower than that of 0·5 μM phenylephrine in the absence of TEA. This may explain why the control response to acetylcholine in the presence of TEA was no less than that in the absence of TEA, although the EDHF-mediated response is inhibited in the presence of TEA.

Daunorubicin at the higher concentration of 50 μM and aclarubicin (20 μM) strongly attenuated the relaxing response to acetylcholine, regardless of whether TEA was present. Also, they markedly inhibited the increase of tissue cGMP in response to acetylcholine. On the other hand, the endothelium-independent relaxing response to sodium nitroprusside, from which NO is liberated and activates soluble guanylate cyclase in vascular smooth muscle (Waldman & Murad 1987), remained unchanged even in the presence of 50 μM daunorubicin and 20 μM aclarubicin, and could not be reversed by 50 μM daunorubicin. In the previous study, we showed that aclarubicin inhibits endothelium-dependent relaxation by acting somewhere distal to receptor stimulation in the vascular endothelium (Wakabayashi et al 1991). Thus, daunorubicin at 50 μM, like aclarubicin, does inhibit the NO-mediated relaxation in response to acetylcholine. The inhibitory effect of 20 μM aclarubicin on the relaxation and increase in tissue cGMP in response to acetylcholine was stronger than that of 50 μM daunorubicin (Fig. 1, Table 1). Moreover, even in the strip pretreated with 50 μM daunorubicin, 20 μM aclarubicin could reverse the relaxing tension induced by acetylcholine to above the precontractile level (Fig. 2A, B). Thus, aclarubicin has a more potent inhibitory action on the NO-mediated vasorelaxation than daunorubicin.

The difference between the daunorubicin and aclarubicin effects on the EDRF-mediated response may arise from differences in their chemical structures. A recent study by Hirano et al (1991) has shown that pirarubicin (1·5–45 μM), an anthracycline analogue, causes endothelium-dependent relaxation of rat isolated aorta. They proposed that the tetrahydropyranyl group in anthracycline analogues plays an important role in producing endothelium-dependent relaxation of rat aorta. Daunorubicin, aclarubicin and pirarubicin possess the basic structure of a tetracyclic quinoid aglycone, of which C-7 is linked to L-daunosamine (daunorubicin), L-cinerulose-(1→4)-2-deoxy-L-fucose-(1→4)-L-rhodosamine (aclarubicin) or tetrahydropyranyl-(1→4)-L-daunosamine (pirarubicin) (Di Marco et al 1965; Hirano et al 1991). We found that aclarubicin at 5·9–23·6 μM or daunorubicin at 50 μM inhibits the endothelium-dependent relaxation. The difference in the effects on the EDRF-mediated response among the anthracycline analogues may be due to differences in their side chains.

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