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Inhibitory effects of a fullerene derivative, dimalonic acid C_{60}, on nitric oxide-induced relaxation of rabbit aorta

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Abstract

Dimalonic acid C_{60} (10^{-5} M), a new fullerene derivative, produced an augmentation of phenylephrine-induced tone and reduced both the acetylcholine-induced maximum relaxation and the amplitude of substance P (10^{-8} M)-induced relaxation in endothelium-containing thoracic aorta of rabbit; the acetylcholine- and substance P-induced relaxation was restored in the presence of superoxide dismutase (250 U/ml). Dimalonic acid C_{60} (10^{-5} M) did not influence the phenylephrine-induced contractile response in the absence of endothelium, but the acetylcholine-induced relaxation was eliminated by removal of the endothelium. Superoxide anion generation, using hypoxanthine (1 mM)/xanthine oxidase (16 mU/ml), reduced the acetylcholine-induced relaxation and produced an augmentation of phenylephrine-induced tone in endothelium-containing strips; these effects were negated by the addition of superoxide dismutase (250 U/ml). A nitric oxide-generating agent, *S*-nitroso-*N*-acetylpenicillamine, caused relaxation of aorta without endothelium in a concentration-dependent manner, and the concentration-response curve was shifted to the right in the presence of dimalonic acid C_{60}. This inhibitory effect of dimalonic acid C_{60} was also masked in the presence of superoxide dismutase. Sodium nitroprusside-induced relaxation was not affected by either dimalonic acid C_{60} or superoxide dismutase. These observations suggest that dimalonic acid C_{60} inhibits endothelium (nitric oxide)-dependent agonist-induced relaxation through the production of superoxide.

Keywords: Smooth muscle; Endothelium; Dimalonic acid C_{60}; Nitric oxide (NO); Superoxide dismutase

1. Introduction

Endothelium-derived relaxing factor (EDRF) is a potent vasodilator produced by vascular endothelial cells. Many investigators (Gryglewski et al., 1986; Moncada et al., 1986; Palmer et al., 1987) have reported that nitric oxide (NO) mimics the effect of EDRF, and that EDRF is identical to NO, as evidenced by the results of bioassays and pharmacological techniques, chemiluminescence measurements or immunochemical procedures. NO is a highly reactive radical ($NO^{\cdot}$) in physiological solution, and interacts with oxygen radicals ($O_2^{-\cdot}$) to produce a cytotoxic oxidant, peroxynitrite (Beckman et al., 1990). This potential for loss of NO-induced relaxation and increased production of peroxynitrite following the reaction of NO with superoxide anion contributes to a number of pathological situations such as hypertension (Wei et al., 1985), ischemia-reperfusion injury (Downey, 1990), diabetes (Langenstroer and Pieper, 1992) and cytotoxic brain injury (Lipton et al., 1993). In addition to this pathological role of NO, the production of NO in vascular endothelium is controlled by a large number of biological mediators such as acetylcholine (Furchgott and Zawadzki, 1980) and substance P (Enokibori et al., 1994), and by the physical shearing force of flowing blood (Rubanyi and Vanhoutte, 1986). In a recent report Mian and Martin (1995) suggested that the basal activity of NO is more sensitive than acetylcholine-induced NO activity to inactivation by superoxide anion, and that the degradation of NO is reduced by endogenous superoxide dismutase.

Fullerene (C_{60}), a condensed ring aromatic compound with extended π systems (Kratschemer et al., 1990), is a

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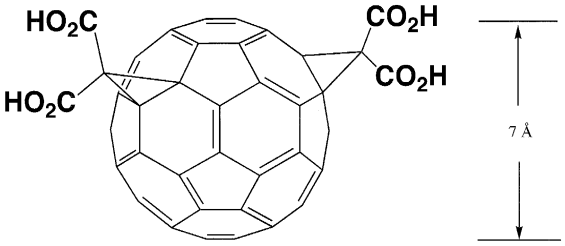

Fig. 1. Structure of dimalonic acid C_{60}.

novel carbon allotrope (Kroto et al., 1985). C_{60}, which has a unique structure chemically and physically, is effectively excited by light irradiation (Arbogast et al., 1991) and can convert molecular oxygen to highly reactive atomic oxygen with a quantum yield of nearly unity (Allemand et al., 1991). C_{60} is soluble in organic solvents but is virtually insoluble in water (Sivaraman et al., 1992; Nakajima-Yamakoshi et al., 1994). Owing to its poor solubility in polar solvents, investigation of the biological and pharmacological properties of fullerene is difficult (Satoh et al., 1995). Although water-soluble C_{60} derivatives have recently been synthesized (Hirsch et al., 1994; Lamparth and Hirsch, 1994), their pharmacological effects have not yet been studied.

We examined the effects of a recently synthesized C_{60} derivative, dimalonic acid C_{60} (Fig. 1), and found that it has a potent and selective inhibitory effect on the endothelium-dependent relaxation induced by agonists and endogenous NO in the vascular system, but does not affect the agonist-induced contractile responses of smooth muscles.

2. Materials and methods

2.1. Drugs

Dimalonic acid C_{60} was synthesized by us with the methods reported by Hirsch et al. (1994) and Lamparth and Hirsch (1994). Diethyl bromomalonate (4.28 mmol) was added to a benzene solution (500 ml) containing C_{60} (0.697 mmol) and 60% NaH (52.8 mmol) under Ar at room temperature. Four hours after the addition of diethyl bromomalonate, 4 ml of 2 M H_2SO_4 was added dropwise to the reaction mixture, which was then dehydrated with anhydrous $MgSO_4$ and the benzene solution was evaporated. $C_{62}(COOEt)_4$ was purified by silica gel chromatography. $C_{62}(COOEt)_4$ (0.225 mmol) and 60% NaH (16.5 mmol) were added in 150 ml dry toluene, then stirred under Ar at 80°C for 10 h. Five ml of MeOH was added dropwise to the reaction mixture, then 150 ml 2 M H_2SO_4 was added. The precipitate was filtered and washed with 2 M H_2SO_4, H_2O, benzene, and CH_2Cl_2. The resulting $C_{62}(COOH)_4$ (dimalonic acid C_{60}) was dried under vacuum for over 12 h at 70°C. Purity of dimalonic acid C_{60} was more than 98%. Other drugs used in the present study were pentobarbital sodium (Abbott Labs, North Chicago, IL, USA), phenylephrine hydrochloride, N^{ω}-nitro-L-arginine (L-NNA), flurbiprofen (Sigma, St. Louis, MO, USA), hypoxanthine, xanthine oxidase, superoxide dismutase, malonic acid (Wako-Junyaku, Osaka, Japan), substance P (Peptide Institute, Osaka, Japan), *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside (Dojindo Laboratories, Kumamoto, Japan). Other chemicals used were of analytical grade.

2.2. Mechanical response

Male albino rabbits weighing 2.0–3.0 kg were anesthetized with an intravenous injection of pentobarbital sodium (50 mg/kg) and killed by bleeding from the carotid arteries. The thoracic aorta was quickly removed and dissected free of excess fat and connective tissue in oxygenated Krebs solution of the following composition (in mM): NaCl, 118; $MgCl_2$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25 and glucose, 11.0 dissolved in distilled water (pH 7.4 at 37°C). The thoracic aortae were cut into helical strips about 10 mm in length and 2 mm in width. For the endothelium-denuded preparations, the endothelial cells were removed by gently rubbing the aorta strip with a cotton probe, and the functional loss of endothelial cells was confirmed by loss of the relaxation response to acetylcholine (10^{-6} M) after phenylephrine precontraction. The physiological solution also contained propranolol (10^{-6} M), yohimbine (3×10^{-7} M), desmethylimipramine (10^{-7} M) and normetanephrine (10^{-6} M) to block β- and α_2-adrenoceptors and to inhibit neural and nonneural uptake of catecholamines, respectively.

2.3. Analysis of mechanical responses

Cumulative concentration-response curves of agonists were obtained. Agonistic activity was expressed as a pD_2 value, which is the negative logarithm of the molar concentration required to produce 50% of the maximum response to the drug (EC_{50}). In an antagonist experiment, the strips were equilibrated with dimalonic acid C_{60} for 20 min after determination of control concentration-response curves, and the curves were then obtained in the presence of dimalonic acid C_{60} in the same preparation.

2.4. Statistics

Numerical results are expressed as the means ± S.E., and statistical significance was calculated by Student's *t*-test or Duncan's new multiple range test. A P value less than 0.01 was considered to indicate significant difference.

3. Results

3.1. Effect of dimalonic acid C_{60} on acetylcholine- or substance P-induced relaxation in rabbit thoracic aorta

Endothelium-containing preparations of thoracic aorta were contracted by phenylephrine (10^{-6} M), and the contraction lasted for at least 3 h. The phenylephrine-induced contraction was relaxed by acetylcholine in a concentration-dependent manner (Fig. 2A). The pD_2 value for acetylcholine was 6.69 ± 0.13 ($n = 9$), and the amplitude of the maximum relaxation induced by acetylcholine was $66.9 \pm 1.7\%$ ($n = 4$) of the phenylephrine-induced sustained contraction. This acetylcholine-induced relaxation was completely eliminated in the presence of dimalonic acid C_{60} (10^{-5} M) (Fig. 2B), and an acetylcholine-induced contractile response was observed at the concentration of 10^{-5} M. As shown in Fig. 2D and Table 1, dimalonic acid C_{60} concentration dependently reduced the maximum amplitude of the acetylcholine-induced relaxation without significantly changing the pD_2 values obtained from the concentration-response curves. With the addition of superoxide dismutase (250 U/ml) the acetylcholine-induced relaxation was restored (Fig. 2C and E). As shown in Fig. 3A–D, the application of 10^{-8} M substance P also produced transient relaxation of the phenylephrine-induced contraction, and the magnitude of the relaxation was $41.3 \pm 0.4\%$ ($n = 14$) of the phenylephrine-induced sustained contraction. Substance P-induced relaxation was significantly reduced by pretreatment with dimalonic acid C_{60} (10^{-5} M), and the relaxation was restored in the presence of superoxide dismutase, as summarized in Fig. 3E. Malonic acid (10^{-4} M) did not affect either the phenylephrine-induced contraction or the acetylcholine-induced relaxation, and dimalonic acid C_{60} (10^{-6}–3×10^{-5} M) had no direct effect on smooth muscle. We also observed that the NO synthetase inhibitor, L-NNA (10^{-4} M) completely eliminated the acetylcholine-induced relaxation (data not shown).

3.2. Effect of hypoxanthine / xanthine oxidase on acetylcholine-induced relaxation

These experiments were conducted in the presence of catalase (3000 U/ml) to prevent the actions of hydrogen peroxide. The superoxide anion-generating enzyme and its substrate, hypoxanthine (1 mM)/xanthine oxidase (16 mU/ml), produced an augmentation of phenylephrine-induced tone in endothelium-containing preparations. The magnitude of the augmentation was $7.70 \pm 2.64\%$ ($n = 4$).

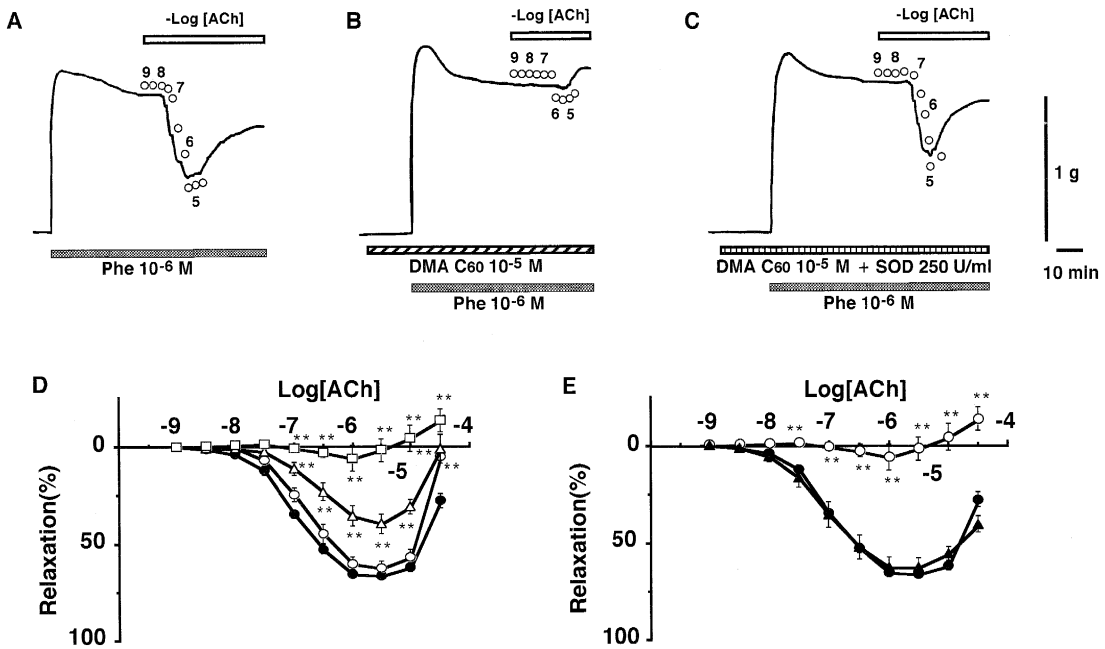

Fig. 2. Typical traces showing the relaxation effect elicited by acetylcholine (ACh) (A), the inhibitory effect of dimalonic acid C_{60} (DMA C_{60}) on ACh-induced relaxation (B) and the effect of superoxide dismutase (SOD) on the inhibitory effect of DMA C_{60} (C) in phenylephrine-contracted endothelium-containing strips of rabbit thoracic aorta. (D) Concentration-dependent inhibition of DMA C_{60} on the concentration-response curves of ACh-induced relaxation. (E) Restoration of the ACh-induced relaxation by the addition of SOD. In A, B and C, ACh was cumulatively added to the preparation after phenylephrine precontraction. Phenylephrine (10^{-6} M) was applied as indicated by the gray bar, DMA C_{60} (10^{-5} M) as indicated by the hatched bar, and DMA C_{60} (10^{-5} M) and SOD (250 U/ml) as indicated by the striped bar. In D, the abscissa is the log concentration (M) of ACh, and the ordinate is the percent relaxation, with 100% representing the phenylephrine-induced sustained contraction. ●, ACh alone; ○, ACh with 10^{-6} M DMA C_{60}; △, ACh with 3×10^{-6} M DMA C_{60}; □, ACh with 10^{-5} M DMA C_{60}. In E, the abscissa is the log concentration (M) of ACh, and the ordinate is the percent contraction of which 100% represents ACh-induced sustained contraction. ●, ACh alone; ○, ACh with 10^{-5} M DMA C_{60}; ▲, ACh with 10^{-5} M DMA C_{60} and 250 U/ml SOD. Each value is the mean ± S.E. (vertical bars) of 4 experiments. ** Significantly different from value obtained from the DMA C_{60}-untreated preparation ($P < 0.01$).

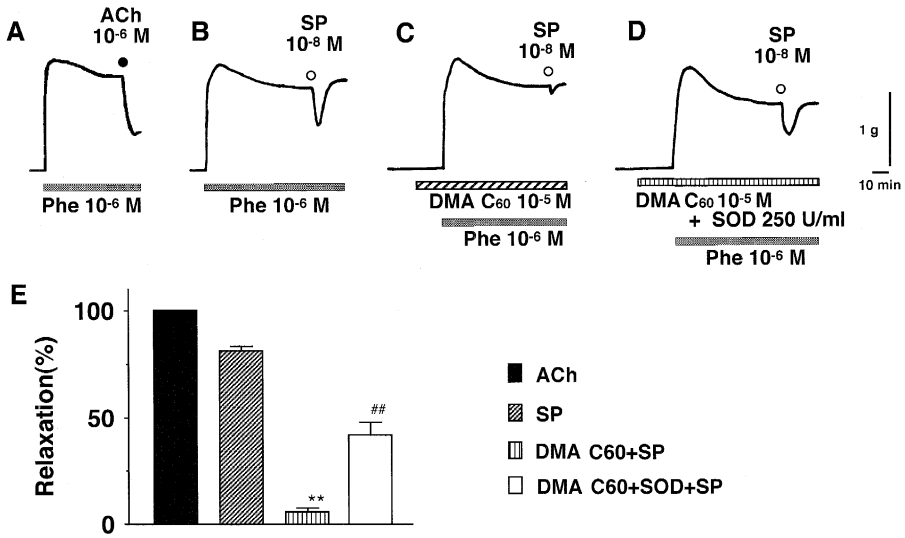

Fig. 3. Typical traces of the relaxation effects of acetylcholine (A) and substance P (B), the inhibitory effect of dimalonic acid C_{60} (DMA C_{60}) on substance P-induced relaxation (C) and the restoration of acetylcholine-induced relaxation by superoxide dismutase (SOD) after inhibition by DMA C_{60} (D) in phenylephrine-contracted endothelium-containing strips of rabbit thoracic aorta. (E) Relaxation amplitudes. Phenylephrine (Phe, 10^{-6} M) was applied as indicated by the gray bar, DMA C_{60} (10^{-5} M) as indicated by the hatched bar, and DMA C_{60} (10^{-5} M) and SOD (250 U/ml) as indicated by the striped bar. In E, the ordinate is the percent relaxation of which 100% represents acetylcholine-induced relaxation. Each value is the mean ± S.E. (vertical bars) of 4 experiments. ** Significantly different from the value obtained from the untreated preparation ($P < 0.01$). ## Significantly different from the value obtained from the DMA C_{60}-treated preparation ($P < 0.01$).

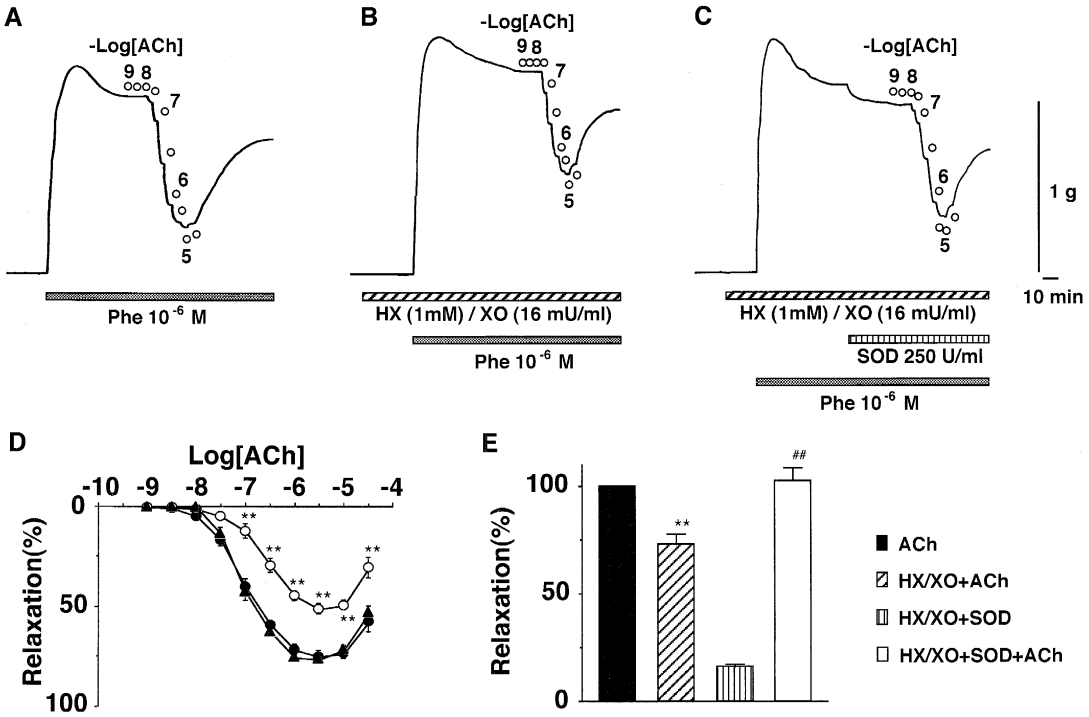

Fig. 4. Typical traces of the relaxation effect of acetylcholine (A), the inhibitory effect of hypoxanthine/xanthine oxidase (B) and the effect of superoxide dismutase (SOD) on the hypoxanthine/xanthine oxidase-treated preparation (C) in phenylephrine-contracted endothelium-containing strips of rabbit thoracic aorta. (D) Concentration-response curves obtained from A, B and C. (E) Relaxation amplitudes. Phenylephrine (Phe, 10^{-6} M) was applied as indicated by the gray bar, hypoxanthine (HX, 1 mM)/xanthine oxidase (XO, 16 mU/ml) as indicated by the hatched bar, and HX (1 mM)/XO (16 mU/ml) and SOD (250 U/ml) as indicated by the striped bar. In D, the abscissa is the log concentration (M) of ACh, and the ordinate is the percent relaxation, with 100% representing phenylephrine-induced sustained contraction. ●, ACh alone; ○, ACh with 1 mM HX/16 mU/ml XO; ▲, ACh with 1 mM HX/16 mU/ml XO and 250 U/ml SOD. In E, the ordinate is the percent relaxation of which 100% represents acetylcholine-induced relaxation. Each value is the mean ± S.E. (vertical bars) of 4 experiments. ** Significantly different from the value obtained from the untreated preparation ($P < 0.01$). ## Significantly different from the value obtained from the HX/XO-treated preparation ($P < 0.01$).

Table 1
Effects of dimalonic acid C_{60} (DMA C_{60}) on acetylcholine (ACh)-, *S*-nitroso-*N*-acetylpenicillamine (SNAP)- and sodium nitroprusside (SNP)-induced relaxation and the protective effect of superoxide dismutase (SOD) in phenylephrine-contracted strips of rabbit thoracic aorta

	n	pD_2 value relaxation (%)	Maximum
ACh			
ACh alone	4	6.69 ± 0.13	66.9 ± 1.7
$+3\times10^{-6}$ M DMA C_{60}	4	6.63 ± 0.08	40.0 ± 4.8 [a]
$+10^{-5}$ M DMA C_{60}	4	6.46 ± 0.04	6.1 ± 6.7 [a]
$+10^{-5}$ M DMA C_{60} + 250 U/ml SOD	4	7.09 ± 0.11	63.2 ± 5.0
SNAP			
SNAP alone	11	6.67 ± 0.04	98.9 ± 0.27
$+10^{-5}$ M DMA C_{60}	4	5.66 ± 0.04 [a]	78.3 ± 1.17 [a]
$+10^{-5}$ M DMA C_{60} + 250 U/ml SOD	5	6.46 ± 0.05	97.2 ± 3.91
SNP			
SNP alone	4	7.73 ± 0.02	98.4 ± 2.97
$+10^{-5}$ M DMA C_{60}	4	7.23 ± 0.01	100.0 ± 2.78
$+10^{-5}$ M DMA C_{60} + 250 U/ml SOD	6	7.20 ± 0.04	96.8 ± 0.79

The relaxation responses induced by ACh were obtained from endothelium-containing aortic strips. The relaxation responses induced by SNAP and SNP were obtained from endothelium-denuded strips. Maximum relaxation is expressed as a percentage; 100% represents phenylephrine-induced sustained contraction. Values are given as the means ± S.E.
[a] Significantly different from the value obtained with DMA C_{60}-untreated preparations ($P < 0.01$).

The maximum amplitude of acetylcholine-induced relaxation was decreased by hypoxanthine/xanthine oxidase in endothelium-containing preparations (Fig. 4B). Treatment with superoxide dismutase (250 U/ml) partially relaxed the phenylephrine-induced sustained contraction, and restored the acetylcholine-induced relaxation in the presence of hypoxanthine/xanthine oxidase (Fig. 4C). The magnitude of the acetylcholine- and superoxide dismutase-induced relaxation is shown in Fig. 4E.

3.3. Effect of dimalonic acid C_{60} on S-nitroso-N-acetylpenicillamine- and sodium nitroprusside-induced relaxation

As shown in Fig. 5A, in endothelium-denuded preparations, the NO-generating agent, *S*-nitroso-*N*-acetylpenicillamine, caused relaxation to the baseline in a concentration-dependent manner, and the pD_2 value for this agent was 6.71 ± 0.05 ($n = 4$). The concentration-response curves for *S*-nitroso-*N*-acetylpenicillamine were shifted to the right in the presence of dimalonic acid C_{60} (10^{-5} M), and this inhibitory effect of dimalonic acid C_{60} was prevented by the presence of superoxide dismutase (250 U/ml). By contrast, the NO mimetic agent sodium nitroprusside induced relaxation in a concentration-dependent manner, an effect that was not affected by either dimalonic acid C_{60} (10^{-5} M) or superoxide dismutase (250 U/ml) (Fig. 5B). The data obtained from these experiments are summarized in Table 1.

4. Discussion

This study showed that dimalonic acid C_{60} does not affect receptors mediating contraction and relaxation of muscle, but that it does cause specific inhibition of acetylcholine- and substance P-induced relaxation in endothelium-containing thoracic aorta and of *S*-nitroso-*N*-acetylpenicillamine-induced relaxation in endothelium-denuded thoracic aorta of rabbit. Dimalonic acid C_{60} thus does not prevent agonist-receptor binding or receptor function (it is not a receptor antagonist), nor does it affect the contractile or relaxation mechanisms of muscle; however, it does

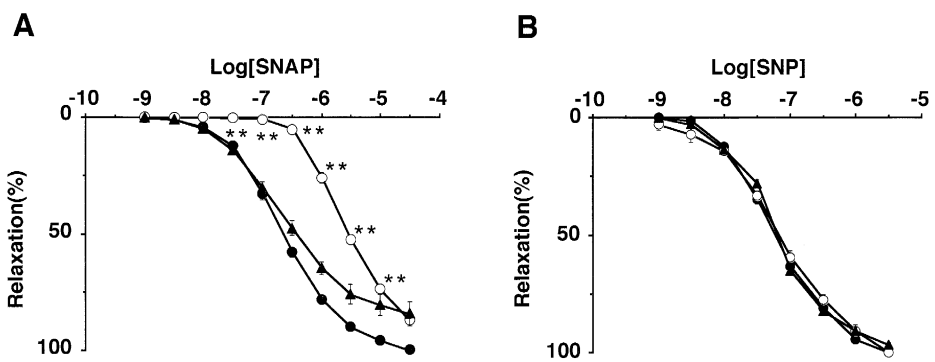

Fig. 5. Effects of dimalonic acid C_{60} (DMA C_{60}) and superoxide dismutase (SOD) on *S*-nitroso-*N*-acetylpenicillamine (SNAP) (A)- and sodium nitroprusside (SNP) (B)-induced relaxation in phenylephrine-contracted endothelium-denuded strips of rabbit thoracic aorta. In A, the abscissa is the log concentration (M) of SNAP, and the ordinate is the percent relaxation of which 100% represents phenylephrine-induced sustained contraction. In B, the abscissa is the log concentration (M) of SNP, and the ordinate is the percent relaxation of which 100% represents phenylephrine-induced sustained contraction. ●, control; ○, with 10^{-5} M DMA C_{60}; ▲, with 10^{-5} M DMA C_{60} and 250 U/ml SOD. Each value is the mean ± S.E. (vertical bars) of 4 experiments. ** Significantly different from the value obtained from the DMA C_{60}-untreated preparation ($P < 0.01$).

inhibit NO-induced relaxation via the degradation of NO after it has reacted with superoxide.

Acetylcholine-induced relaxation of vascular smooth muscle is dependent on endogenous NO derived from the endothelium. We also tested whether acetylcholine- and substance P-induced relaxation was abolished by the removal of the endothelium or by treatment with the NO synthase inhibitor L-NNA (100 μM). In the present experiment, dimalonic acid C_{60} prevented acetylcholine-induced relaxation of the sustained contraction produced by phenylephrine in endothelium-containing strips of rabbit thoracic aorta. As shown in Fig. 2D and Table 1, the amplitude of the maximum relaxation induced by acetylcholine was reduced, but the pD_2 value was not affected, by application of dimalonic acid C_{60}. Superoxide dismutase prevented the inhibition exerted by dimalonic acid C_{60}, suggesting that inhibition of acetylcholine-induced relaxation involves the generation of superoxide (Fig. 2C and D). These results are consistent with reports that NO is inactivated by superoxide generated in physiological solution and that superoxide dismutase reduces the inactivation of NO (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Palmer et al., 1987). In endothelium-containing preparations, substance P also produced relaxation, which was reduced by the application of dimalonic acid C_{60} and was restored by treatment with superoxide dismutase (Fig. 3). Though we have only limited information on the chemical properties of dimalonic acid C_{60} in physiological systems, these findings suggest that the inhibition caused by dimalonic acid C_{60} is due to the acceleration of NO destruction by highly reactive molecules such as superoxide anion.

In the present experiment, we found differences between the activity of dimalonic acid C_{60} and other superoxide-generating systems. Acetylcholine-induced relaxation was significantly reduced in the presence of hypoxanthine/xanthine oxidase (Fig. 4B and D), which is also a superoxide-generating system (Hyslop and Sklar, 1984), but the magnitude of the decrease was only approximately 25%. Furthermore, the phenylephrine-induced contraction increased in the presence of hypoxanthine/xanthine oxidase, and treatment with superoxide dismutase partially relaxed the phenylephrine-induced sustained contraction and restored the acetylcholine-induced relaxation. Dimalonic acid C_{60} completely inhibited the acetylcholine-induced relaxation (Fig. 2E) that was significantly larger than that produced by hypoxanthine/xanthine oxidase (Fig. 4D). The difference in the sensitivity of the acetylcholine-induced relaxation to hypoxanthine/xanthine oxidase and dimalonic acid C_{60} may be explained by the reports that basal NO is free, whereas NO released in response to agonists comes from a pre-formed intracellular store (Ignarro, 1991; Cocks and Angus, 1991). As in previous studies (Wei et al., 1985; Rubanyi and Vanhoutte, 1986; Abrahamsson et al., 1992), the inhibition of acetylcholine-induced relaxation by hypoxanthine/xanthine oxidase likely occurred as a consequence of destruction of NO by superoxide anion since it was blocked by superoxide dismutase. Thus, it appears that higher concentrations of superoxide were required to destroy agonist-stimulated NO than spontaneous NO. One explanation for these findings may be that endogenous superoxide dismutase protects agonist-induced NO from destruction by superoxide (Mian and Martin, 1995). However, there is not enough evidence to explain the differences between the effects of hypoxanthine/xanthine oxidase and dimalonic acid C_{60} in the present experiment. Further analytical studies investigating the differences between basal- and agonist-induced NO are required to understand fully the differences between the effects of hypoxanthine/xanthine oxidase and dimalonic acid C_{60}.

In vascular endothelium cells, muscarinic cholinoceptor stimulation causes Ca^{2+} channel opening and NO synthesis. NO is enzymatically synthesized from the terminal guanidino-nitrogen of L-arginine by NO synthases, yielding L-citrulline as a co-product in the presence of intracellular Ca^{2+} (Palmer et al., 1988; Marletta, 1993). The produced NO diffuses into the vascular smooth muscle cells, activates soluble guanylate cyclase and elevates intracellular cyclic GMP, which is involved in relaxation (Gruetter et al., 1979). In addition to NO, the *S*-nitrosothiols *S*-nitroso-*N*-acetylpenicillamine and *S*-nitrosoglutathione mimic the relaxation induced by NO in denuded preparations of aorta (Ignarro et al., 1981; Kowaluk et al., 1987; Kowaluk and Fung, 1990), suggesting that these *S*-nitrosothiols donate NO. Kowaluk and Fung (1990) reported that vascular smooth muscle exhibits substantial catalytic activity for NO generation from *S*-nitroso-*N*-acetylpenicillamine, and suggested that denitration of *S*-nitrosothiols is not spontaneous and that it is catalyzed at external vascular membranes. In the present experiment, NO derived from *S*-nitroso-*N*-acetylpenicillamine may have been produced by enzymes in the cell membranes of vascular smooth muscle. As shown in Fig. 5A, the relaxation induced by *S*-nitroso-*N*-acetylpenicillamine was inhibited by dimalonic acid C_{60}, and this inhibitory effect of dimalonic acid C_{60} was prevented by the presence of superoxide dismutase. By contrast, NO production from another type of NO-generating substance, sodium nitroprusside, is facilitated by the cytochrome P-450 system in vascular smooth muscle cells (Bennett et al., 1992), suggesting that sodium nitroprusside-derived NO is produced inside the muscle cells and activates soluble guanylate cyclase at the same time. These different NO-producing mechanisms may explain the different sensitivities to dimalonic acid C_{60}, that is, endothelium- and *S*-nitroso-*N*-acetylpenicillamine-derived NO is degraded by superoxide produced by dimalonic acid C_{60} but sodium nitroprusside-derived NO is not affected by superoxide.

In summary, we found for the first time that dimalonic acid C_{60} has a strong biological activity; superoxide is generated by the fullerene and inhibits muscle relaxation

mediated by NO. Among the numerous implications of these findings, the most exciting prospect is the use of dimalonic acid C_{60} as a new type of pharmacological tool.

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