*Br. J. Pharmacol.* (1988), **95**, 1015–1022

# Age-associated decrease in histamine-induced vasodilatation may be due to reduction of cyclic GMP formation

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**1** The effects of aging on histamine-induced vasodilatation and cyclic GMP production in rat thoracic aorta were investigated.

**2** This histamine-induced dilatation of the aorta was mediated by $H_1$-receptors and was dependent on the endothelium.

**3** Histamine induced the greatest dilatation of arteries of 3–4 week old rats, progressively less of those of rats of 8 to 56 weeks old, and scarcely detectable dilatation of those of 100 week old rats.

**4** Histamine induced cyclic GMP production in aorta from rats of 4 weeks old, with no change in the cyclic AMP level. This increase in the cyclic GMP level induced by histamine also decreased with age, being about 70% as great at 8 weeks, 50% as great at 50–60 weeks, and 10% as great at 130 weeks of age.

**5** Removal of the endothelium completely abolished the histamine-induced increase in cyclic GMP.

**6** The dilator effect of nitroprusside, which enhances cyclic GMP production by stimulating guanylate cyclase directly (not indirectly via the endothelium derived relaxing factor, EDRF), also showed age-related attenuation.

**7** The dilator effect of 8-bromo cyclic GMP, which stimulates cyclic GMP-dependent protein kinase, also decreased during aging.

**8** These results suggest that aging reduces the ability of the endothelium to produce EDRF, which stimulates guanylate cyclase, and so decreases cyclic GMP production. Thus the decreased dilator response of the arteries to histamine during aging is probably due to both loss of endothelial function and reduction of guanylate cyclase activity. Alteration of cyclic GMP-dependent protein kinase activity may also participate in the age-related changes.

## Introduction

Vasodilatation induced by $\beta$-agonists has been found to decrease age-dependently in animals (O'Donnell & Wanstall, 1984; Tsujimoto *et al.*, 1986; Hyland *et al.*, 1987; Sawyer & Docherty, 1987), and this change has been suggested to be due to decreased adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production (Schoeffter & Stoclet, 1983; Tsujimoto *et al.*, 1986). We have shown that the dilator response of rat mesenteric artery to histamine, which is mediated by $H_1$-receptors and is endothelium-dependent, is attenuated or lost with an increase in the age of rats (Moritoki *et al.*, 1986). However, the reason for this decrease in dilator response to histamine is still unknown. The endothelium of blood vessels has been shown to be important in regulating vascular tone and the dilator responses to substances such as acetylcholine, histamine and bradykinin (Furchgott, 1983; Rapoport & Murad, 1983a; Van de Voorde &

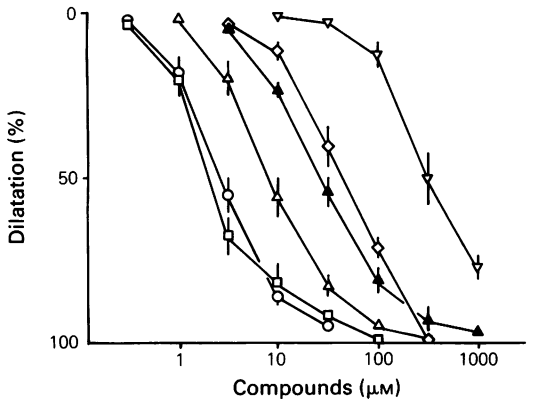


**Figure 1** Effects of chlorpheniramine and cimetidine on histamine-induced dilatation, and dilator effects of 2-pyridylethylamine and 4-methylhistamine on the thoracic aorta from 8 week-old rats. The aortae were treated with 1 μM noradrenaline. (○) Histamine alone; (△) histamine with 10 nM chlorpheniramine; (▲) histamine with 30 nM chlorpheniramine; (□) histamine with 100 μM cimetidine; (◇) 2-pyridylethylamine; (▽) 4-methylhistamine. Vasodilatations are expressed as percentages of noradrenaline (1 μM)-induced contraction. Values are means of preparations from 5 rats; vertical lines indicate s.e.mean.

Leusen, 1983; Moritoki *et al.*, 1986). Moreover, it has been found that histamine increases the guanosine 3':5'-cyclic monophosphate (cyclic GMP) level, thus causing dilatation (Rapoport & Murad, 1983a).

Therefore, in this work we examined whether the decrease with age in the dilator response of rat thoracic aorta to histamine is due to an age-dependent decrease in endothelium function or cyclic GMP production. For further clarification of the step(s) affected by aging, the ability of nitroprusside to dilatate the arteries and induce cyclic GMP formation, and the dilator effect of 8-bromo cyclic GMP were examined in aortic preparations from rats of various ages.

A preliminary account of some of these data was presented at the 6th International Symposium on Vascular Neuroeffector Mechanisms, Melbourne, 1987 (Moritoki, 1987).

## Methods

### *Organ bath experiments*

Male Wistar rats aged 3 weeks (19–24 days, 122 ± 22 g), 4 weeks (28–32 days, 139 ± 22 g), 8 weeks (234 ± 3 g), 12 weeks (±1 week, 419 ± 14 g), 50–63 weeks (537 ± 17 g), 100 weeks (90–105 weeks, 486 ± 12 g) and 130 weeks (125–132 weeks, 590 ± 19 g) were used. The rats were killed by a blow to the head and cervical dislocation, and bled. Segments of thoracic aorta, 4 mm in length, were cut with parallel blades and freed of adhering tissue under a dissecting microscope. Ring segments were then set up in a 10 ml organ bath containing Krebs solution of the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. The medium was maintained at 34°C and bubbled with 95% $O_2$ and 5% $CO_2$. The preparations of arteries from rats of 3–4, 8–12 and 50–130 weeks old were equilibrated for 2 h under resting tensions of 0.5, 1.0 and 1.3 g, respectively. Responses were recorded isometrically with a force displacement transducer (Nihon Kohoden SB-1TH).

For measurement of dilator responses, the arteries were first treated with 0.3–1 μM noradrenaline, which produced about 80 to 90% of the maximal contraction. Drugs were added cumulatively to the bath in a volume of 10–200 μl, and dilator responses were plotted as percentages of the noradrenaline-induced contractions. Endothelium function was confirmed by testing acetylcholine-induced dilatation. For investigation of the function of the vascular endothelium, the lumen of the arteries was rubbed with cotton threads to remove the endothelium. Loss of endothelial function was confirmed by checking the loss of a dilator response to acetylcholine without loss of the response to verapamil.

### *Assay of cyclic nucleotides*

Segments of aorta were equilibrated in Krebs solution bubbled with 95% $O_2$ and 5% $CO_2$ for 2 h before the addition of 0.3 μM noradrenaline. Then the preparations were incubated with histamine or nitroprusside. After various times of incubation with histamine or nitroprusside, the preparations were quickly frozen in liquid nitrogen and then homogenized in ice-cold 6% trichloroacetic acid in a Potter glass-glass homogenizer on ice. The homogenates were centrifuged at 1700 *g* for 15 min at 4°C, and the supernatants were extracted with 3 volumes of water-saturated ether. Cyclic nucleotides (cyclic GMP or cyclic AMP) were measured by a radioimmunoassay procedure (Honma *et al.*, 1977) using commercially available kits. Briefly, cyclic nucleotides in the supernatant were succinylated and incubated with [$^{125}$I]-succinyl cyclic GMP tyrosine methyl ester or [$^{125}$I]-succinyl cyclic AMP tyrosine methyl ester and antisera for 18 h at 4°C. Then dextran-coated charcoal was added to the reaction medium to terminate the reaction. The radioactivity in the supernatant was counted in a gamma spec-

trometer. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Each experimental group consisted of 4 to 6 preparations from different rats. Values are expressed as means ± s.e.means. The statistical significance of differences was analysed by Student's unpaired *t* test, and *P* values of less than 0.05 were considered to be significant.

The materials used were histamine dihydrochloride, nitroprusside, 8-bromo cyclic GMP, papaverine hydrochloride, verapamil hydrochloride, noradrenaline bitartrate, chlorpheniramine maleate, methylene blue, hydroquinone and phenidone (Sigma Chemical Co., St Louis, MO), cimetidine, 4-methyl histamine and 2-(2-pyridyl)ethylamine (Smith Kline & French Laboratories, Philadelphia, PA) and kits for radioimmunoassay of cyclic GMP and cyclic AMP (Yamasa Shoyu Co., Choshi, Japan).

## Results

### *Characteristics of histamine-induced dilatation*

Histamine at concentrations above 0.3 μM caused dilatation of thoracic aorta from 8-week-old rats that had been treated with noradrenaline (1 μM) to produce tone (Figures 1 and 2). The histamine-induced dilatation was not affected by concentrations of up to 100 μM of the $H_2$-antagonist cimetidine (Figure 1), but was inhibited by concentrations of 10–30 μM of the $H_1$-antagonist chlorpheniramine (Figure 1). The $H_2$-selective agonist 4-methylhistamine was about 10 fold less potent as an inhibitor as the $H_1$-selective agonist 2-pyridylethylamine. Removal of the endothelium by rubbing the lumen of the aorta with cotton threads completely abolished the dilator response of the arteries to histamine (at up to 10 μM) and acetylcholine (at up to 1 μM), without affecting the response to papaverine ($pD_2$ values, 5.82 with and 5.72 without endothelium) and verapamil ($pD_2$ values, 5.90 with and 5.67 without endothelium). Addition of methylene blue at concentrations (0.3–3 μM) which did not affect noradrenaline-induced contractions, reversed the maximal dilatation induced by 100 μM histamine to the level before histamine addition. Similar reversals of the effect of histamine were observed with hydroquinone (10 μM) and phenidone (1 μM).

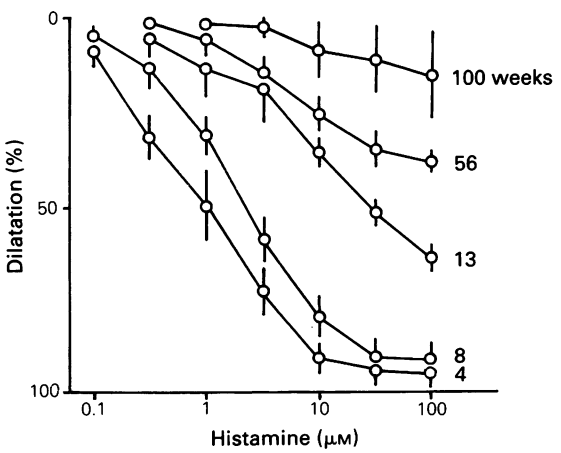


**Figure 2** Effect of aging on the dilator response of rat thoracic aorta to histamine. The arteries were contracted with noradrenaline at concentrations corresponding to $EC_{80}$ values (0.3 μM at 4 weeks old and 1.0 μM at 8 weeks or more). Histamine was added cumulatively. Dilator responses are expressed as percentages of the noradrenaline-induced contraction. Ages of rats are shown in weeks. Values are means of preparations from 6 rats; vertical lines indicate s.e.mean.

### *Effect of aging on histamine-induced dilatation*

Dilatations of 93.6 ± 3.1% and 91.3 ± 5.4% ($n = 6$) by 100 μM histamine were observed with preparations of thoracic aorta from rats of 4 and 8 weeks old, respectively. With an increase in age of the rats, the response to histamine decreased. As shown in Figure 2, with preparations from rats of 13 and 56 weeks old, the dilatations induced by 100 μM histamine were 63.9 ± 4.5% and 38.6 ± 2.1%, respectively, and aortae of 100-week-old rats showed scarcely any response to histamine.

In contrast, no effect of aging was seen on the dilator responses of the aorta to verapamil ($pD_2$ value, 6.30 ± 0.05 at 3 weeks and 6.15 ± 0.08 at 20–22 weeks, $n = 4$) or papaverine ($pD_2$ value, 5.31 ± 0.06 at 4 weeks and 5.08 ± 0.11 at 15 weeks, $n = 4$).

### *Effect of aging on cyclic GMP formation*

The time course of cyclic GMP formation in the arteries of rats of 12 weeks old induced by 100 μM histamine is shown in Figure 3. Histamine caused a transient increase in the cyclic GMP level to a maximum of 2.74 ± 0.39 pmol $mg^{-1}$ protein ($n = 5$) after 15 s followed by a rapid decrease to the basal level within 2 min. This increase in cyclic GMP level was completely abolished by removal of the endothelium from the arteries (Figure 3). On the basis of the time course of cyclic GMP formation, the dose-dependence of the effect of histamine on preparations from 8 week-old rats was examined after incubation with histamine for 15 s. In the presence of

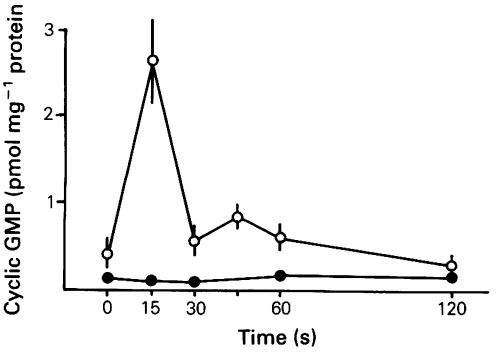


**Figure 3** Time course of the change in cyclic GMP levels in rat thoracic aorta induced by 100 $\mu$M histamine in the presence and absence of endothelium. Arteries from rats of 12 weeks old were incubated with the $EC_{80}$ concentration of noradrenaline for 5 min before exposure to histamine. (○) Amounts of cyclic GMP in preparations with endothelium and (●) those in preparations without endothelium. Amounts of cyclic GMP at or below threshold level in preparations without endothelium were considered to be 0.1 pmol $mg^{-1}$ protein. The symbols represent means of values in 5 arteries from 9 rats; vertical lines indicate s.e.mean.

1, 10, 100 and 1000 $\mu$M histamine, the cyclic GMP level increased from the control value of $0.94 \pm 0.48$ pmol $mg^{-1}$ protein to $1.60 \pm 0.27$, $3.22 \pm 0.36$, $3.72 \pm 0.64$ and $3.33 \pm 0.85$ pmol $mg^{-1}$ protein ($n = 5$), respectively.

To determine whether the age-related attenuation of the histamine-induced dilator response was associated with a reduction of cyclic GMP formation, we examined the histamine (100 $\mu$M)-stimulated production of cyclic GMP at 15 s in preparations from rats of various ages. As shown in Figure 4, accumulation of cyclic GMP in 15 s was $6.26 \pm 1.37$ pmol $mg^{-1}$ protein ($n = 6$) in aortae of rats of 4 weeks old, but in the aortae from rats of 12, 50–60 and 130 weeks old, its accumulation decreased to $3.18 \pm 0.55$, $1.86 \pm 0.32$ and $0.50 \pm 0.08$ pmol $mg^{-1}$ protein, respectively.

*Effect of aging on cyclic AMP formation*

In contrast to the marked increase in the cyclic GMP level, the cyclic AMP levels in the aortae of rats of 4 ($2.43 \pm 0.25$ pmol $mg^{-1}$ protein), 12 ($2.29 \pm 0.24$ pmol $mg^{-1}$ protein) and 50–60 ($2.19 \pm 0.46$ pmol $mg^{-1}$ protein) weeks old were not increased after 30 s incubation with 100 $\mu$M histamine ($2.55 \pm 0.14$ pmol $mg^{-1}$ protein at 4 weeks, $2.35 \pm 0.19$ pmol $mg^{-1}$ protein at 12 weeks and $2.47 \pm 0.44$ pmol $mg^{-1}$ protein at 50–60 weeks old, $n = 4$–9), and these levels were not age-dependent.

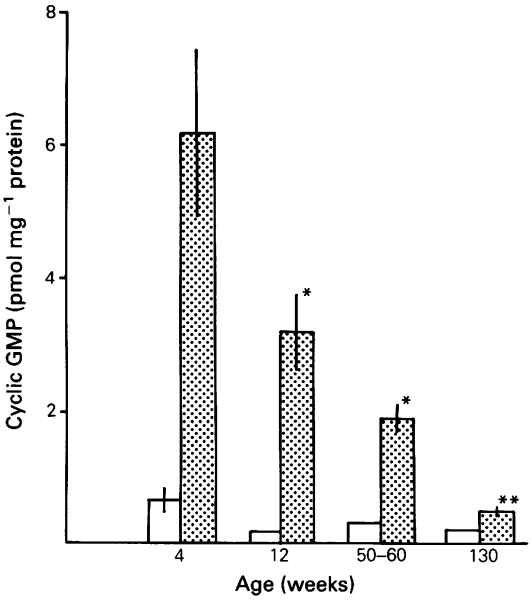


**Figure 4** Effect of aging on the increase in the cyclic GMP level in aorta induced by 100 $\mu$M histamine. Arteries were incubated with the $EC_{80}$ concentrations of noradrenaline before exposure to histamine. Amounts of cyclic GMP were measured after incubation with histamine for 15 s. Stippled columns, histamine-induced cyclic GMP accumulation; open columns, spontaneous cyclic GMP accumulation. Each column represents the mean of values in preparations from 5 to 8 rats; vertical lines indicate s.e.mean. $*P < 0.05$, $**P < 0.01$, compared with value at the preceding age (unpaired $t$ test).

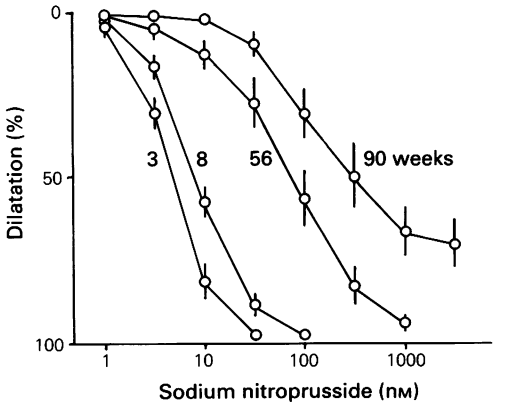


**Figure 5** Effect of aging on nitroprusside-induced dilatation of rat thoracic aorta. The arteries were treated with the $EC_{80}$ concentration of noradrenaline to produce tone. Vasodilatations are expressed as percentages of the noradrenaline-induced contraction. Ages of rats are shown in weeks. Values are means of preparations from 5 animals; vertical lines indicate s.e.mean.

*Age-dependent effect of nitroprusside*

The dilatation caused by nitroprusside was not dependent on the endothelium and was reversed by methylene blue (0.3 $\mu$M). This dilator response also decreased age-dependently, the dose-response curve shifting in parallel to the right with increase in age of rats (Figure 5).

In the aorta of 8 week-old rats, nitroprusside at a concentration of 1 $\mu$M stimulated the formation of cyclic GMP to a maximum of $122.8 \pm 7.5$ pmol mg$^{-1}$ protein ($n = 7$; Figure 6), which was attained after incubation with nitroprusside for 60 s and was maintained for more than 2 min. This stimulation of cyclic GMP production was not endothelium-dependent: in preparations without endothelium, cyclic GMP accumulation was $95.0 \pm 14.9\%$ ($n = 3$) of that of paired, normal preparations ($n = 2$). With an increase in age of rats to 130 weeks, this cyclic GMP formation decreased greatly to about 5% of that at 8 weeks (Figure 6).

*Age-dependence of effect of 8-bromo cyclic GMP*

Aging significantly ($P < 0.05$) decreased the dilator response of the aorta to 8-bromo cyclic GMP: the dose-response curve for 8-bromo cyclic GMP shifted 3 fold to the right with an increase in age from 4 weeks ($pD_2$ value, $4.75 \pm 0.09$, $n = 4$) to 130 weeks ($pD_2$ value, $4.09 \pm 0.11$, $n = 4$) (Figure 7).

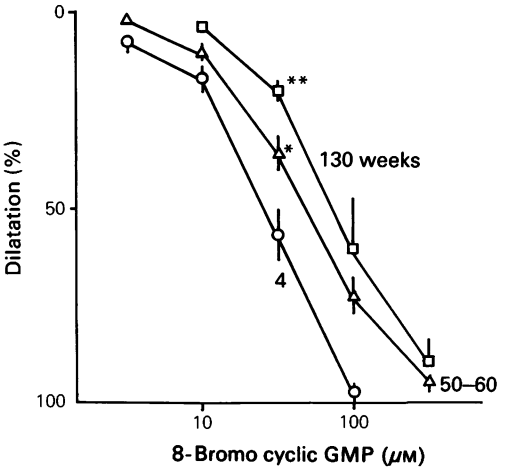


**Figure 7** Effect of aging on dilatation of rat aorta induced by 8-bromo cyclic GMP. Arteries were incubated with the $EC_{80}$ concentration of noradrenaline (0.3 $\mu$M for 4 weeks old and 1 $\mu$M for over 8 weeks old) to produce tone. Dilatations are expressed as percentages of noradrenaline-induced contraction. Ages of rats are shown in weeks. Points are means of values for the arteries of 5 rats; vertical lines indicate s.e.mean. $*P < 0.05$, $**P < 0.01$, compared with the value for the preparations from 4 week-old rats (unpaired $t$ test).

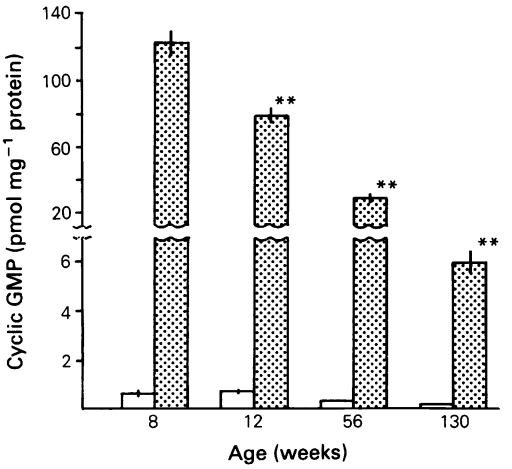


**Figure 6** Effect of aging on the increase in the cyclic GMP level induced by nitroprusside (1 $\mu$M). Cyclic GMP was measured after incubation with the $EC_{80}$ concentrations of noradrenaline for 5 min and then with and without nitroprusside for 60 s. Stippled and open columns represent amounts of cyclic GMP in the presence and absence of nitroprusside, respectively. Columns show means of values of preparations from 5 rats; vertical lines indicate s.e.mean. $**P < 0.01$, compared with value at the preceding age (unpaired $t$ test).

## Discussion

In this work the dilator response of rat thoracic aorta to histamine was confirmed to be endothelium-dependent and to be mediated by $H_1$-receptors (Van de Voorde & Leusen, 1983), like that of rat mesenteric artery (Moritoki *et al.*, 1986). In addition, stimulation of guanylate cyclase by endothelium-derived relaxing factor (EDRF; Ignarro *et al.*, 1986) seemed to be involved in the histamine-induced vasodilatation, because methylene blue (Gruether *et al.*, 1981; Martin *et al.*, 1985) and hydroquinone and phenidone (Griffith *et al.*, 1984; Moncada *et al.*, 1986) abolished the dilatation. The histamine-induced dilatation of the aorta decreased with an increase in the age of rats. This age-related decrease was not specific to histamine, because it was also observed with other dilatators such as nitroprusside, and has been found to occur in $\beta$-agonist-induced dilatation of rat aorta (Schoeffter & Stoclet, 1982; 1983; Tsujimoto *et al.*, 1986; Sawyer & Docherty, 1987). Therefore, the age-related alteration seems to occur at some step(s) beyond the receptor.

Histamine and acetylcholine are known to act on the endothelium of vessels inducing production and liberation of EDRF, which in turn activates soluble guanylate cyclase in vascular smooth muscle, and as a result increases cyclic GMP production, and so triggers dilatation (Rapoport & Murad, 1983a; Griffith *et al.*, 1985; Ignarro *et al.*, 1986). Therefore, the age-related decrease in the histamine-induced dilator response could be due to (1) a reduction in number or affinity of $H_1$-receptors, (2) a functional loss of the endothelium, (3) a decrease in guanylate cyclase activity, (4) a decrease in cyclic GMP-dependent protein kinase activity, and, or (5) an increase in cyclic GMP phosphodiesterase activity.

A reduction in the number or affinity of $H_1$-receptors in the endothelium, as has been demonstrated for $\beta$-adrenoceptors in rat heart (Bhalla *et al.*, 1980), is considered to be a cause of the decrease in the dilator response to histamine. However, in the rat aorta, $H_1$-receptors are localized in the endothelium and so we did not examine whether they show a change in number or affinity in binding assays using tritiated $H_1$-antagonists. Histamine is known to induce EDRF production by the endothelium through $H_1$-receptors, and this EDRF directly activates soluble guanylate cyclase in vascular smooth muscle (Rapoport & Murad, 1983a), as does acetylcholine, bradykinin and arachidonic acid (Ignarro *et al.*, 1986).

In the present experiments, we found that endothelium-dependent production of cyclic GMP in the arteries in histamine-stimulated states was greater in young rats than in old rats. Moreover, after removal of the endothelium, histamine at up to 100 $\mu$M did not induce a dilatation of arteries or an increase in the cyclic GMP level (Figure 3). Therefore, the primary defect responsible for the age-related decrease in histamine-mediated dilatation may be a decrease in the ability of endothelium to produce EDRF, or a reduction in the number or affinity of $H_1$-receptors. At present it is uncertain which of these steps is impaired by aging, but in either case our results suggest that the endothelium, which plays an important role in mediating vascular homeostasis, loses functional activity not only on mechanical injury but also during aging.

Aging may also affect the dilator response to histamine at a level beyond the endothelium. The EDRF induced by vasodilators is proposed to be a direct activator of soluble guanylate cyclase, and to generate cyclic GMP (Förstermann *et al.*, 1986; Ignarro *et al.*, 1986). Vasodilatation caused by nitrocompounds such as nitroprusside is associated with an increase in the vascular level of cyclic GMP (Rapoport & Murad, 1983b; Ignarro & Kadowitz, 1985), bypassing the endothelium-dependent vasodilator system and causing direct activation mainly of soluble guanylate cyclase in the vessels. Furthermore, an age-dependent decrease in the dilator response to nitroprusside has been demonstrated (Sawyer & Docherty, 1987). Therefore, using nitroprusside, we examined whether aging affects the activity of guanylate cyclase. We demonstrated that the effects of nitroprusside both in dilating rat aorta and in elevating the cyclic GMP level were also attenuated by aging. Moreover, we found that the cyclic GMP level did not decrease after mechanical damage of the endothelium. These results indicate that age-related changes also occurred at the level of guanylate cyclase or a later step(s).

The accumulation of cyclic GMP, whether triggered by histamine or nitroprusside, may induce vasodilatation through stimulation of cyclic GMP-dependent protein kinase, which in turn phosphorylates some protein or dephosphorylates the myosin light chain (Rapoport *et al.*, 1982; 1983; Rapoport & Murad, 1983a), or activates the calcium extrusion pump to lower intracellular calcium (Popescu *et al.*, 1985). To determine whether cyclic GMP-mediated steps are affected by aging, we examined the dilator effect of 8-bromo cyclic GMP. The dilatation induced by 8-bromo cyclic GMP was significantly ($P < 0.05$) reduced by aging. Therefore, the possibility of age-related changes at the level of cyclic GMP-dependent protein kinase or at steps beyond the protein kinase, cannot be ruled out.

Age-associated increases in phosphodiesterase activity at low substrate concentrations has been demonstrated (Lugnier & Stoclet, 1979). If an increase in cyclic GMP phosphodiesterase activity is responsible for the age-associated decrease of the dilator responses to histamine and nitroprusside, then high concentrations of histamine should increase the cyclic GMP level and cause dilatation, especially in preparations from aged rats, by overcoming the degradation of cyclic GMP by the phosphodiesterase. But this was not the case; in the arteries from rats of 100 weeks old, histamine did not induce dilatation at any concentration tested.

The concentration ranges of histamine necessary to produce cyclic GMP and dilate the aorta were similar. However, histamine-induced dilatation persisted for a long time, and during this period an increase in the cyclic GMP level occurred only transiently. Dissociation of the time courses of dilatation and elevation of the cyclic GMP level could be explained by a slower turnover of a step activated by cyclic GMP rather than by that of cyclic GMP itself. In this respect, it is noteworthy that nitroprusside-induced dilatation and protein phosphorylation are mediated after the elevated cyclic GMP level has declined (Rapoport *et al.*, 1982).

In contrast to the finding that a reduction in the cyclic AMP level during aging is responsible for the

loss of β-agonist-induced vasodilatation (Schoeffter & Stoclet, 1982; 1983; Tsujimoto *et al.*, 1986), the present results showed that histamine did not elevate the cyclic AMP level, suggesting that cyclic AMP is not involved in the age-related alteration in the histamine-induced dilator response.

Our results suggest that the endothelium and guanylate cyclase are affected by aging, but it is still unknown to what extent they are affected.

We conclude from this work that the age-associated loss of the dilator response of rat aorta to histamine is probably mainly due to gradual losses of both endothelium function and guanylate cyclase activity. Moreover, since the dilator effect of 8-bromo cyclic GMP was attenuated by aging, alteration of cyclic GMP-dependent protein kinase activity with age may also be involved in this phenomenon.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (60,571,048). We are grateful to Smith Kline & French Laboratories, Philadelphia, PA, for supplying cimetidine, 4-methylhistamine and 2-pyridylethylamine.

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*(Received December 4, 1987*
*Revised June 9, 1988*
*Accepted June 28, 1988)*