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Reduction of endothelial function with age in the mesenteric arterial bed of the normotensive rat

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1 Age-related changes in endothelial vasodilator function were studied in an *in vitro* preparation of the mesenteric arterial bed removed from male, normotensive, Wistar rats.

2 Animals were killed at 2, 12 or 22 months of age, the superior mesenteric artery was cannulated and the gut removed. The mesenteric arterial bed was perfused at a constant flow rate of 4 ml min^{-1} and perfusion pressure was taken as an index of arteriolar tone.

3 The muscarinic agonist, carbachol, antagonized noradrenaline-induced vasoconstriction in the presence, but not in the absence, of endothelium. This cholinoceptor agonist-induced release of endothelial-derived relaxing factor (EDRF) was impaired in 22 month old rats.

4 Noradrenaline-induced vasoconstriction increased following removal of endothelium suggesting that agonist-induced release of EDRF attenuates vasoconstrictor responses to noradrenaline measured in the presence of endothelium.

5 Removal of endothelium had less effect on noradrenaline-induced vasoconstriction in old rats suggesting once again that agonist-induced release of EDRF is impaired in old rats.

6 The noradrenaline dose-response curve established in the presence of endothelium was shifted to the left in 22 month old rats.

7 In conclusion, aging in the rat appears to lead to a reduction in endothelial vasodilator function in a resistance vessel.

Keywords: Aging; endothelium; noradrenaline; rat; mesenteric arterial bed.

Introduction

Vasoconstrictor mechanisms in response to noradrenaline are impaired with age (Tuttle, 1966; Elliott *et al.*, 1982; Simpkins *et al.*, 1983; Soltis *et al.*, 1984; Fouda & Atkinson, 1986; Hyland *et al.*, 1987; Wanstall & O'Donnell, 1988; Johnson & Wray, 1990). Furthermore several (Shimizu & Toda, 1986; Luscher *et al.*, 1993) but not all (Hynes & Duckles, 1987) reports suggest that agonist induced release of endothelium-derived relaxing factor (EDRF) also decreases with age. This parallel fall in noradrenaline and EDRF responsiveness is paradoxical. Noradrenaline-induced release of EDRF attenuates vasoconstriction to noradrenaline (Miller *et al.*, 1987), so an age-related decrease in EDRF release could counterbalance any age-related decrease in the sensitivity to noradrenaline. If this were to be the case the vascular responsiveness to noradrenaline would be apparently unaltered. This is suggested by reports from several groups (Frolkis *et al.*, 1975; Scott & Reid, 1982; Hyland & Docherty, 1985; Hynes & Duckles, 1987). Furthermore, several reviews have stressed that the results on the evolution of α-adrenoceptor vasoconstrictor mechanisms with age are inconsistent (Fleisch, 1980; Kelly & O'Malley, 1984; Bennett & Gardiner, 1988; Vanhoutte, 1988). We would suggest that part of this inconsistency lies in the functional state of the endothelium of the preparations in which vasoconstriction in response to noradrenaline is measured.

Our objective was, therefore, to investigate whether responses to noradrenaline and agonist-induced release of EDRF evolve in a similar fashion in aging rats. The mesenteric arterial bed was used as an *in vitro* model of a resistance vessel network. Preparations were removed from 2-, 12- and 22-month old, male normotensive, Wistar rats. Vasoconstrictor responses to noradrenaline were recorded in the presence and absence of endothelium. Endothelium was removed by perfusing the arterial bed with air for a short period. Pharmacomechanical responses produced by noradrenaline were compared to those produced electromechanically by depolarizing solutions of potassium chloride.

Vasodilator endothelial function was evaluated from the degree of antagonism by carbachol of vasoconstriction in response to noradrenaline. In the mesenteric arterial bed of the rat, carbachol antagonizes noradrenaline-induced vasoconstriction via a mechanism which is dependent on EDRF release, but independent of prostaglandin release (Randall & Hiley, 1988). Responses to carbachol were compared to those evoked by sodium nitroprusside, which vasodilates via a mechanism which does not rely upon release of EDRF (Murphy *et al.*, 1990).

Methods

Animals

Experiments were performed in outbred, Wistar, male rats (IOPS AF/Han, Iffa-Credo, L'Arbresle, France) of 2, 12 or 22 months of age, kept under standard conditions in the animal house for ≥ 1 week before experiments were performed.

The mesenteric arterial bed preparation

Animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). The superior mesenteric artery was cannulated and the gut removed (McGregor, 1965; Henrion *et al.*, 1991). The mesenteric arterial bed was perfused at 4 ml min^{-1} with modified Krebs bicarbonate solution (mM: NaCl 118, KCl 4.7, $MgCl_2.6H_2O$ 1.2, NaH_2PO_4 1.0, $CaCl_2.2H_2O$ 2.6, $NaHCO_3$ 25 and glucose 11.1; 37°C; pH 7.35–7.45), oxygenated with a 95% O_2 and 5% CO_2

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mixture. A bubble trap system, with a flow rate of 0.2 ml min^{-1}, removed any air bubbles in the perfusate.

The flow rate was fixed at 4 ml min^{-1}, a rate we have previously used for mesenteric arterial bed preparations from adult Wistar rats (Henrion *et al.*, 1991). Arteriolar vasoconstriction produced an increase in perfusion pressure (mmHg) which was measured with a strain gauge transducer (Beckman, Palo Alto, CA, U.S.A.), placed in the perfusion circuit just before the mesenteric arterial bed. Mean perfusion pressure was recorded after electronic integration of the pulsatile pressure signal. All pressure values given were corrected by subtraction of the pressure generated by the tubing of the perfusion system (= 4 to 6 mmHg). Baseline perfusion pressure was between 9 and 12 mmHg.

After a 15 min stabilization period, noradrenaline was injected (0.6 μg in a bolus of 0.33 ml, during 20 s) into the perfusate, at 5 min intervals from 15 to 35 min (see Table 1), by which time a reproducible vasoconstrictor response was obtained. Protocols were based on the repeated injection of a bolus of noradrenaline every 5 min. This allowed us to check that the reactivity of each individual preparation had returned to normal following each intervention.

Preliminary experiment in 12-month old rats: evolution of noradrenaline responses in the presence and absence of endothelium

This experiment was performed in 12-month old rats only. Following equilibration and stabilization of responses (see above), noradrenaline was injected every 5 min up to the 160th min ($n = 6$). A second group followed a similar protocol except that following equilibration and stabilization, endothelium was removed by passing air bubbles through the perfusion system (0.2 ml min^{-1} for 5 min), by inversing the direction of flow in the bubble trap system. Noradrenaline was then injected every 5 min up to the 160th min ($n = 6$).

General protocol

This was split up into 5 consecutive stages (Table 1): (i) attenuation by carbachol of noradrenaline-induced vasoconstriction, (ii) noradrenaline dose-response curve, (iii) removal of endothelium, (iv) potassium chloride dose-response curve, and (v) attenuation by sodium nitroprusside of noradrenaline-induced vasoconstriction. At the end of each of the 5 stages, the vasoconstrictor response to the noradrenaline bolus was measured.

Responses in the presence of endothelium

Firstly, the mesenteric bed was perfused for 5 min with carbachol (10^{-9} M), at the end of which time a noradrenaline bolus was injected in the presence of carbachol. The protocol was repeated with stepwise increases in the concentration of carbachol up to 10^{-4} M. The antagonism by carbachol of noradrenaline-induced contraction, is expressed as maximal (%) and IC_{50} i.e., the concentration of carbachol required to reduce the vasoconstriction induced by noradrenaline in the absence of carbachol, by half (following (logit [response])/(log_{10} [dose] transformation)). The midrange sensitivity is estimated from the IC_{50} values for antagonists and the ED_{50} values for agonists, i.e., if IC_{50} or ED_{50} increase then midrange sensitivity is said to decrease and *vice versa*.

Secondly, a noradrenaline dose-response curve was constructed by injecting noradrenaline (0.33 ml in 20 s, 56 ng to 56 μg) into the perfusion system every 5 min. Responses to noradrenaline are expressed as: (i) maximal increases in perfusion pressure (perfusion pressure, mmHg), and (ii) midrange sensitivity, estimated from ED_{50}, i.e., the dose required to produce an increase in perfusion pressure equivalent to 50% of the maximal value (following (logit [response])/(log_{10} [dose] transformation)).

Thirdly, following perfusion with Krebs bicarbonate solution for 15 min, the endothelium was removed. The absence of endothelium was checked by perfusing carbachol (10^{-5} M) for 5 min then injecting a noradrenaline bolus. Carbachol failed to attenuate noradrenaline-induced vasoconstriction in arterial beds without endothelium in all groups (results not shown).

Responses in the absence of endothelium

Fourthly, a dose-response curve for potassium chloride was constructed by injecting increasing concentrations of potassium chloride (1–5 mg, 0.33 ml in 20 s) every 5 min. Responses to potassium chloride are expressed as maximum and EC_{50}.

Fifthly, sodium nitroprusside (10^{-9} M) was perfused for 5 min, at the end of which time noradrenaline was injected. The protocol was repeated with stepwise increases in the concentration of sodium nitroprusside up to 10^{-4} M. Antagonism by sodium nitroprusside of vasoconstriction to noradrenaline is expressed as maximum and IC_{50}.

There were 15 rats in each age group.

Drugs used

Carbachol chloride, and sodium nitroprusside were purchased from Sigma, St. Louis, MO, U.S.A. Noradrenaline bitartrate was dissolved in 0.1 mM ascorbic acid. Concentrations or doses are given as base. Sodium pentobarbitone was purchased from Sanofi SA, Paris, France. Noradrenaline bitartrate and other chemicals were purchased from Merck AG, Darmstadt, Germany.

Statistics

Results are expressed as means ± s.e.mean. Experiments were performed according to a randomized block design. Significant differences between means were determined either by ANOVA and Scheffe test, or Student's paired t test. Results from linear regression ANOVA are given as origin = a, slope = b, correlation coefficient = r. The probability level chosen was $P < 0.05$ (NS = not significant).

Table 1 Experimental protocol

Time (min)	*Operation*
0–15	Equilibration
15–35	Stabilization of responses to 5 challenges with NA (0.6 μg)
40–65	Physiological antagonism of NA-induced vasoconstriction by carbachol
70–100	NA dose-response curve
110	Removal of endothelium
115–135	Potassium chloride dose-response curve
135–160	Physiological antagonism of NA-induced vasoconstriction by sodium nitroprusside

NA = noradrenaline

Results

Rats weighed 295 ± 2 g at 2 months, 513 ± 6 g at 12 months and 625 ± 15 g at 22 months of age.

Preliminary experiment in 12-month old rats: evolution of noradrenaline responses in the presence and absence of endothelium

Responses in the presence of endothelium were stable whereas those determined in the absence of endothelium decreased linearly with time (Figure 1).

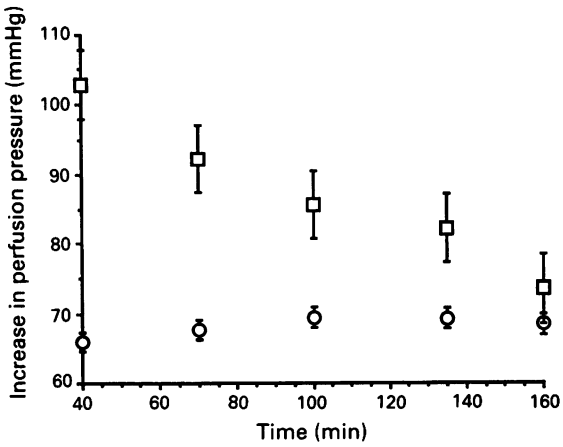

Figure 1 Evolution of noradrenaline responses in the presence (○) and absence (□) of endothelium, in mesenteric arterial bed preparations removed from 12-month old Wistar rats ($n = 6$ per group). Noradrenaline (0.6 μg) was injected every 5 min but results are given for times 40, 70, 100, 135 and 160 min only (see Table 1). In the absence of endothelium (□) there was a significant fall in responses (Y) with time (X): a = 110, b = − 0.22, $r = 0.981$, $P < 0.05$.

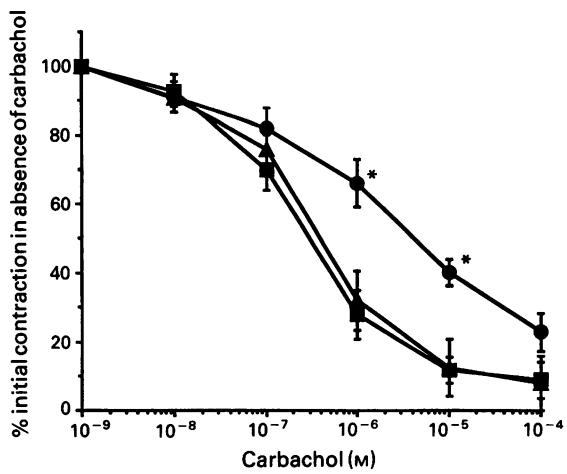

Figure 2 Effect of age on physiological antagonism by carbachol of noradrenaline-induced vasoconstriction in the mesenteric arterial bed (in the presence of endothelium) from 2-month old (■), 12-month old (▲) and 22-month old rats (●). *$P < 0.05$ versus 2-month old rats (■). Initial vasoconstrictor responses to a noradrenaline bolus (0.6 μg) in the absence of carbachol were 47 ± 4, 59 ± 8 and 86 ± 6 mmHg in 2-, 12- and 22-month old rats, respectively.

Responses in the presence of endothelium

Carbachol ($\leq 10^{-4}$ M) had no effect on baseline perfusion pressure (results not shown), but antagonized vasoconstriction to noradrenaline in a dose-related manner (Figure 2). IC_{50} and maxima for carbachol were 0.21 ± 0.05 μM and 90 ± 6%, 0.42 ± 0.05 μM and 93 ± 2%, and 5.00 ± 0.12 μM ($P < 0.05$ versus 2-month old) and 78 ± 3% in 2-, 12- and 22-month old rats.

Noradrenaline EC_{50} was lower in 22-month old rats (0.3 ± 0.1 μg) than in 2-month old rats (1.0 ± 0.2 μg, $P < 0.05$, Figure 3). Maximal responses were similar in all groups (2-month old: 158 ± 12 mmHg).

Removal of endothelium and responses to single bolus injections of noradrenaline

Responses to noradrenaline increased in 2- (54 ± 5 and 90 ± 6 mmHg, paired t test, $P < 0.05$) and 12-month old rats

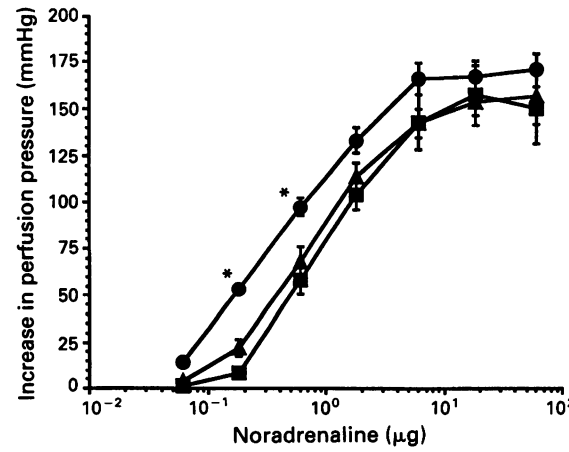

Figure 3 Noradrenaline dose-response curves in mesenteric arterial beds (in the presence of endothelium) from 2-month old (■), 12-month old (▲) and 22-month old rats (●). *$P < 0.05$ versus 2-month old rats (■); $n = 15$ per group.

(65 ± 6 and 95 ± 4 mmHg, paired t test, $P < 0.05$) but not in 22-month old rats (89 ± 6 and 110 ± 7 mmHg, paired t test, NS), following removal of endothelium.

Responses in the absence of endothelium

There was a slight decrease in midrange sensitivity to potassium chloride in 12- (2.13 ± 0.03 mg, $P < 0.05$) and 22-month old rats (2.23 ± 0.03 mg, $P < 0.05$) compared to 2-month old rats (1.95 ± 0.03 mg). Maximal responses were similar (2-month old: 110 ± 6 mmHg).

Sodium nitroprusside had no effect on basal perfusion pressure (results not shown), but antagonized vasoconstriction to noradrenaline in a dose-related manner. Vasoconstrictor responses to noradrenaline (0.6 μg) before sodium nitroprusside were 93 ± 6, 96 ± 4 and 94 ± 5 mmHg in 2-, 12- and 22-month old rats. IC_{50} values for sodium nitroprusside were similar in all groups (2-month old 0.55 ± 0.20 μM), but maximal responses were significantly reduced in 12- (74 ± 2%, $P < 0.05$) and 22-month old rats (73 ± 4%, $P < 0.05$) compared to 2-month old rats (93 ± 4%).

Discussion

We describe several lines of evidence which suggest that endothelial vasodilator function is impaired in the mesenteric arterial bed of 22-month old rats. Firstly, physiological antagonism by carbachol of noradrenaline-induced vasoconstriction was less marked in mesenteric arterial beds from 22-month old rats. Secondly, antagonism by the endothelium of the vasoconstrictor response to a bolus of noradrenaline decreased in 22-month old rats. An experimental consequence of the latter was that the dose-response curve for noradrenaline, constructed in the presence of endothelium, was shifted to the left. This occurred in spite of the fact that the underlying vasoreactivity to noradrenaline, measured in the absence of endothelium, was unchanged, as far as could be judged from responses to a bolus administration. Furthermore, electromechanical vasoconstrictor responses (elicited by potassium depolarization) decreased. Thus when vasoconstrictor responses to noradrenaline were measured in the *in vitro* preparation of the rat mesenteric arterial bed, before removal of the endothelium, an apparent age-related increase in sensitivity to noradrenaline was observed.

Our working hypothesis is based upon the fact that the removal of endothelium had a major effect on responses in 2- and 12-month old rats and that there was no time-dependent

increase in the response to a bolus injection of noradrenaline. We checked this in 12-month old rats. Responses in the presence of endothelium were stable throughout the 160 min of the protocol. In the absence of endothelium responses to a repeated bolus administration of noradrenaline were seen to decrease, not increase, with time.

Published reports on the effect of maturation and/or age on vasoconstrictor responses to noradrenaline are contradictory (see Introduction). We would suggest that a partial explanation of this lies in the functional and/or structural state of the endothelium in the different preparations used. In addition, in human isolated vessels, endothelial function may be impaired by age-related vascular pathologies, such as hypertension and atherosclerosis, which confound studies on vascular disease-free aging in man (Bennett & Gardiner, 1988). These considerations would appear to be of less importance in the present study. Blood pressure does not increase with age in the rat strain we used (Lartaud *et al.*, 1993) and the rat has a natural resistance to the development of age-related vascular pathologies seen in man such as atherosclerosis (Gill *et al.*, 1989) and arteriosclerosis following calcium overload (Atkinson, 1992).

Our observation that vasodilator endothelial function decreases with age confirms previous reports (see Introduction). Before considering possible explanations of this, it should be noted that in this and other protocols, the initial response (before carbachol) to a bolus of noradrenaline in 22-month old rats was higher than in younger rats and such differences in vasoconstrictor responses to noradrenaline could have influenced the degree of physiological antagonism by carbachol. The explanation generally given for the age-related decrease in vasodilator endothelial function is based on age-related changes in the function of the endothelial cell. Other functional changes may be involved as in this work and others (Moritoki *et al.*, 1988), there was an age-related attenuation of responses to sodium nitroprusside. This suggests that age-related changes may also occur at the level of guanylate cyclase or a later step. Functional changes may not be the only ones involved. Although on the basis of the bioassay type of protocol used in our experiments, it is difficult to draw conclusions as to the structural or functional origin of the age-related changes in endothelial function, it is possible that the decrease in endothelial function observed was of structural origin. Several studies have shown that aging is associated with intimal thickening and an increase in collagen in the subendothelial layer (Soltis *et al.*, 1984). This could lead to the formation of a barrier, between the endothelium and the smooth muscle cells of the media, which becomes less and less permeable to the passage of endothelial dilator factor(s), with time.

In our experiments the same flow rate was used in mesenteric arterial bed preparations from rats of different body weights. This technique has been adopted in other aging studies using perfused preparations such as the tail artery and the mesenteric arterial bed (Hynes & Duckles, 1987). It should be pointed out that a vascular network with a smaller initial average diameter when perfused at the same constant flow rate will have a greater proportional increase in pressure with the same absolute change in radius than a vascular network with a larger initial average diameter. This could modify maximal responses but in our experiments such responses were generally similar. We observed parallel changes in dose-response curves for carbachol and noradrenaline. We would suggest that this can be taken as evidence of age-related vascular changes which are independent of any methodological artefact linked to the flow rate used. Furthermore we administered the agonist (noradrenaline) in the presence of the physiological antagonist (carbachol or sodium nitroprusside). In other studies the antagonist was added after precontraction with the agonist (Hynes & Duckles, 1987). It should be recalled that in studies of antagonism, the tissue is generally exposed to the antagonist before adding the agonist. Finally, differences in responses to a single bolus of noradrenaline could be due to changes in altered potency and/or in altered maximal response. In our experiments maximal responses to noradrenaline were similar in all three age groups.

In conclusion, when considering possible *in vivo* consequences of these *in vitro* observations, it could be argued that if a diminution of endothelial vasodilator function with age is a common feature of most vascular beds then, in the absence of any compensatory changes in the renal pressure/volume relationship, old Wistar rats should be hypertensive. Although some reports (Bunag & Teravainen, 1991) suggest that Wistar rats may become moderately hypertensive with age, our own studies on rats of the same strain and sex, from the same breeder, show that Wistar rats do not become hypertensive with age (Lartaud *et al.*, 1993). This could mean that: (i) age-related changes in the mesenteric arterial bed are not representative of changes in other vessels; (ii) age-related changes in agonist-induced EDRF mechanisms do not reflect changes in other EDRF mechanisms such as those stimulated by flow or shear stress; (iii) the age-related change in EDRF function is compensated by other functional and/or structural changes in the endothelium or in other organs (such as the kidney).

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