Aging reduces the responsiveness of coronary arteries from male Wistar rats to the vasodilatory action of testosterone

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ABSTRACT

This study was performed to examine whether aging affects the vasodilatory effects of testosterone in the coronary arteries of male rats. Isolated coronary arteries from young mature (3–4 months) and elderly (22–26 months) male Wistar rats were studied in a wire myograph. Contractile function and endothelial function were assessed by measuring vasomotor responses to 10–100 mmol/l KCl, 0.1 mmol/l prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha}) and 10 l mol/l acetylcholine. Sensitivity to the vasodilatory effects of testosterone was assessed by constructing dose–response curves to concentrations between 1 l mol/l and 1 mmol/l testosterone dissolved in water in vessels maximally preconstricted with PGF\textsubscript{2\alpha}. The compliance characteristics of each vessel and serum testosterone levels from each animal were measured. Histological sections of myocardium were examined for differences in coronary artery morphology. Vessels from elderly animals were significantly more resistant to the vasodilatory effects of testosterone than vessels from young animals (P < 0.001 by analysis of covariance). Vessels from elderly animals were also significantly less compliant (7.32 ± 0.43 l mm/mN, compared with 10.99 ± 1.52 l mm/mN in young animals; P = 0.011), and the levels of circulating testosterone in elderly animals were lower, but not significantly so (2.04 ± 0.63 nmol/l compared with 3.88 ± 1.7 nmol/l; P = 0.32). Vessels from elderly animals were less contractile in response to KCl than those from young animals (P = 0.004 by analysis of covariance). There were no significant differences between the two groups in their responses to PGF\textsubscript{2\alpha} or acetylcholine. Thus it is concluded that coronary arteries from elderly rats are significantly less sensitive to the vasodilatory effects of testosterone than those from young animals.

INTRODUCTION

Advanced age and male gender are the two most powerful predictive factors for coronary artery disease in humans [1]. Impaired vasomotor function in atherosclerotic coronary arteries may cause coronary spasm, contributing to myocardial ischaemia, plaque rupture and myocardial infarction [2–4]. Previous animal and human studies have demonstrated disturbances of vasomotor function in resistance vessels from several different vascular beds associated with increased age, even in the absence of overt atherosclerosis [5–11]. These changes have been attributed to decreased production of endothelium-derived relaxing factor, disruption of elastin and collagen fibres, lipid accumulation, and calcification [12]. However, none of these factors appear to be gender-specific, and do not account for the excess risk among men.

Senescence in men is associated with a gradual decline in levels of circulating androgens, by as much as 40% in normal men between the ages of 40 and 90 years, due to relative testicular resistance to gonadotrophin stimulation [13]. This may be of particular importance to the elderly male with coronary artery disease, as supra-
physiological doses of testosterone have been shown to have a potent vasodilatory action in both animal and human models, in vivo and in vitro [14–18]. These effects may translate into clinical benefit, as a number of human studies have demonstrated that administering supplemental doses of testosterone to patients with angina pectoris both improves symptoms and reduces the ischaemic burden [19–25].

The present study was performed to examine whether aging affects the vasomotor sensitivity to testosterone in isolated rat coronary arteries. A decline in the levels of testosterone associated with aging, in addition to changes in sensitivity to its vasomotor effects, may predispose elderly men to clinical cardiac events.

**METHODS**

Groups of 18 young adult (3–4 months old) and 18 elderly (22–26 months old) male Wistar rats were studied. All animals came from the same breeding stock, were housed under identical conditions and were fed on rat chow and water ad libitum. Animals were killed by concussion and cervical dislocation according to U.K. Home Office guidelines. Blood samples were taken from each animal, centrifuged at 405 g for 10 min and frozen at −20 °C until analysis of total testosterone levels by radioimmunoassay on unextracted plasma samples (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA, U.S.A.).

The hearts were immediately dissected free and plunged into chilled physiological saline solution (120 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l CaCl$_2$, 1.17 mmol/l MgSO$_4$, 25 mmol/l NaHCO$_3$, 1.18 mmol/l KH$_2$PO$_4$, 26.9 mmol/l EDTA and 5.5 mmol/l glucose, dissolved in de-ionized water). Left anterior descending coronary arteries were dissected free from the surrounding myocardium and cut into 2.3 mm rings. These rings were then mounted on two 40 μm nickel wires in the organ baths of a wire myograph (Cambustion Ltd, Cambridge, U.K.). Organ baths were filled with physiological saline solution, heated to 37 °C, and bubbled continuously with 95% O$_2$/5% CO$_2$ to maintain the pH at 7.4.

Compliance characteristics were studied by obtaining dynamic diameter–tension curves for each vessel. Starting from a diameter that yielded minimal tension, the vessel was mechanically stretched via separation of the myograph jaws at a rate of 50 μm/s, until the force exerted upon it reached a maximum tension of 25 mN. The myograph jaws were then returned to their original position at the same rate until zero tension was obtained. The $x/y$ value for the diameter–tension curve was calculated over the diameter range 270–370 μm as a measure of dynamic vessel compliance (μm/mN). This diameter range was chosen as it encompasses the resting diameter of the vessels studied and was therefore representative of the in vivo variability in diameter. Each vessel was then loaded to 90% of the diameter required to produce an equivalent intraluminal pressure of 100 mmHg and active tension recordings were carried out according to the methods originally described by Mulvany and Halpern in 1977 [26]. Vessel viability and reproducibility of contractile characteristics were confirmed by performing two contractile responses to 0.1 mmol/l prostaglandin F$_{1\alpha}$ (PGF$_{1\alpha}$) at the beginning of the experimental protocol. Vessels were discarded if the maximal contractile response was $< 1$ mN·mm$^{-1}$, or if the coefficient of variation of the two initial contractions was $> 5\%$.

A total of 18 vessels from elderly animals and 17 vessels from young animals were again maximally precontracted with PGF$_{1\alpha}$. Endothelial function was assessed by examining the response of nine of the vessels from each group to 10 μmol/l acetylcholine. Dose–response curves to water-soluble testosterone (1 μmol/l–1 mmol/l) were constructed using the remaining eight vessels from young rats and nine vessels from elderly rats, maximally precontracted with 0.1 mmol/l PGF$_{1\alpha}$, in order to examine the effects of aging on the vasodilatory response to testosterone. In a further nine vessels from elderly rats and eight vessels from young rats, dose–response curves to 10–100 mmol/l KCl were constructed, in order to examine the effects of aging on smooth muscle contraction.

We used a water-soluble preparation of testosterone which uses a ‘carrier’ molecule to facilitate dissolution. The carrier used is a cyclic oligosaccharide consisting of seven glucopyranose units. This naturally occurring compound has a relatively rigid doughnut-shaped structure, stabilized by intramolecular hydrogen bonding between the C-2 and C-3 hydroxy groups of neighbouring glucopyranose units. Hydrophobic molecules of testosterone are incorporated into the cavity of cyclodextrins by displacing water. This effectively incorporates the testosterone within the cyclodextrin torus. When the water-soluble complex is diluted in aqueous solvent, the process is reversed, thereby releasing the testosterone into solution. We have demonstrated previously that the carrier molecule alone possesses no vasoreactive properties [18].

All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Sections of myocardium were taken for histological analysis to examine for morphological differences associated with aging. These segments were fixed in 10% formalin, and then embedded in wax to allow sections to be cut. Serial sections were stained with haematoxylin and eosin, elastin Van Giesson and Masson Trichrome stains. These sections were then examined visually for any morphological differences between the two groups. Representative transverse sections ($n = 8$ in each group)
were examined using a digital image analyser to gain a quantitative measure of smooth muscle wall thickness. This is expressed as mean muscle layer thickness/lumen diameter, to allow for error introduced by sampling vessels at different points in the coronary bed.

All results are expressed as means±S.E.M. Animal weight, heart weight and vessel diameter were all normally distributed, and were therefore compared using Students t-test. Serum total testosterone levels were compared using the Mann–Whitney U-test for non-parametric data. Responses to testosterone are expressed as a percentage of the maximal contraction. Dose–response curves were compared using analysis of covariance (ANCOVA), with response being the dependent variable, age being a fixed factor, and dose being a covariate. In a number of animals, the serum total testosterone levels were below the minimum detectable limit.

This study was performed under the U.K. Provision of Animals (Scientific Procedures) Act 1986.

RESULTS

Elderly animals were significantly heavier than the young animals [615±21.5 g and 365±11.9 g respectively; means±S.E.M. (n = 17 in each group); P < 0.001]. Hearts from elderly animals were also significantly heavier than those from young animals (1.45±0.05 g and 0.93±0.02 g respectively; n = 17 in each group; P < 0.001). Despite this, the resting internal diameter of the vessels from the young animals (n = 25) was slightly greater than that of those from elderly animals (n = 27): 369±17 μm and 323±12 μm respectively (P = 0.032). Total testosterone levels were higher in the young animals than in the elderly animals (3.88±1.7 nmol/l and 2.04±0.63 nmol/l respectively), but this difference failed to reach statistical significance (P = 0.32). Five of the samples from elderly animals fell below the lowest recordable level of the assay used (0.7 nmol/l), whereas only one sample from the young animals was below this limit.

Vessel wall compliance (measured as change in internal diameter in μm/mN transmural force applied) was lower in the vessels from elderly animals (n = 27) compared with those from young animals (n = 25): 7.32±0.43 μm/mN and 10.99±1.52 μm/mN respectively (P = 0.011).

Coronary artery contractions in response to 0.1 nmol/l PGF$_{2\alpha}$ were similar for the two groups: 2.68±0.36 mN/mm in the vessels from young animals (mean±S.E.M.; n = 17) compared with 2.86±0.26 mN/mm in the vessels from elderly animals (n = 18) (P = 0.636). Endothelial function was intact in all vessels. Dilatation in response to 10 μmol/l acetylcholine was $-0.87±0.18$ and $-0.88±0.25$ mN/mm in vessels from the young and elderly animals respectively (n = 9; P = 0.666) (Figure 1). Responses to 10–100 nmol/l KCl were smaller in the vessels from elderly animals (n = 9) than in those from young animals (n = 8), with maximal contractions of 0.63±0.11 mN/mm and 1.02±0.2 mN/mm respectively (mean±S.E.M.; f = 9.05, P = 0.004 by ANCOVA) (Figure 2). Vessels taken from elderly animals were less sensitive to the vasodilatory effects of testosterone than vessels taken from young animals (n = 9; f = 12.6, P = 0.001) (Figure 3).
Figure 4  Transverse sections through coronary arteries
(a) Vessel from young animal stained with elastin van Giesson stain; (b) vessel from elderly animal stained with elastin van Giesson stain; (c) vessel from young animal stained with haematoxylin and eosin stain; (d) vessel from elderly animal stained with haematoxylin and eosin stain; (e) vessel from young animal stained with Masson Trichome stain; (f) vessel from elderly animal stained with Masson Trichrome stain. Magnification × 160.

Visual examination of histological sections of coronary arteries demonstrated that the vessels from elderly rats were symmetrically thickened within the smooth muscle layer compared with vessels from young rats (Figure 4). This appeared to be due to hypertrophy of the myocytes rather than to an increase in their actual number. These changes did not appear to be an artefact of tortuosity, direction of slice or sampling level. There was also a little more fibrosis surrounding the arteries from elderly rats, and the ‘onion-skin’ adventitia appeared disorganized. There were no consistent differences between the elastic lamina of the two groups. Digital image analysis demonstrated that the muscle layer thickness/lumen diameter was $0.135 \pm 0.012 \, \mu m$ in vessels from the young rats, compared with $0.189 \pm 0.025 \, \mu m$ in vessels from the elderly rats ($P = 0.078$).

The thickening of the smooth muscle layer in arteries from elderly animals seen on visual examination is in
keeping with the measured decrease in vessel wall compliance, and is supported by the quantitative measurements from digital image analysis. However, the results of visual histological analysis are by their nature subjective, and the small number of vessels studied may not be representative of the total sample.

**DISCUSSION**

In the present study we have demonstrated that there is a significant decrease in the sensitivity of rat coronary arteries to the vasodilatory action of testosterone with increasing age, despite normal contractile responses to PGF$_{2\alpha}$ and normal vasodilatory responses to acetylcholine. We also found a decrease in vessel wall compliance, thickening of the vascular smooth muscle cell layer, and a slight decline in the levels of serum total testosterone.

This study was not designed to examine the mechanism by which supraphysiological doses of testosterone cause vasodilatation, or to examine the role of specific aspects of the aging process, such as the development of hypertension, but merely to examine the impact of aging itself on the vasodilatory action of testosterone. It is therefore difficult to speculate on the exact cause of this decline in sensitivity. Although we did not directly examine whether the witnessed difference in the sensitivity to testosterone between vessels from young and aged animals was endothelial-independent, we have demonstrated previously in this model that the vasodilatory action of testosterone is independent of the vascular endothelium [18]. This fact, and our findings of intact endothelial function in these vessels, suggest that it is unlikely that the reduced sensitivity to testosterone is mediated by an age-related decline in endothelial function. This suggestion does, however, require direct verification.

As the vasodilatory effect of testosterone is independent of the vascular endothelium, its effects must therefore be mediated through a direct action on the vascular smooth muscle cells. Could the decreased sensitivity to the vasodilatory effects of testosterone with increasing age merely be a reflection of degeneration of vascular smooth muscle cell function? We demonstrated an increase in vessel wall stiffness and thickening of the smooth muscle cell layer of the arterial wall in the elderly rats, which may suggest degenerative changes of the vascular smooth muscle cell layer. However, despite these findings, and a decrease in sensitivity to the effects of KCl, there was no difference in the responses to PGF$_{2\alpha}$, a more potent contractile agent in this model, suggesting that vascular smooth muscle function is, at most, only slightly disordered in the vessels from elderly animals.

The age-related decline in sensitivity to the vasodilatory action of testosterone seems unlikely, therefore, to be mediated by a generalized decline in endothelial or vascular smooth muscle cell function. The phenomenon may thus be attributable to a more specific effect of aging on a particular receptor or relaxant mechanism, which are as yet undefined and warrant further study.

This decline in sensitivity to the vasomotor effects of testosterone with increasing age may be of clinical importance. Atheromatous plaques in the coronary arteries are commonly eccentrically placed, with a residual arc of disease-free vessel wall [27]. As the pressure drop across a coronary stenosis is inversely proportional to the fourth power of the minimal lumen diameter, compensatory vasodilatation of this disease-free area of the vessel wall plays an important role in influencing symptoms of myocardial ischaemia [28]. Impaired vasomotor function in atherosclerotic coronary arteries may therefore significantly worsen symptoms of angina pectoris, and has also been implicated in myocardial ischaemia, plaque rupture and myocardial infarction [2–4].

Men with coronary artery disease have lower circulating levels of testosterone than similarly aged men with normal coronary arteries [29]. In addition, there is a significant age-related decline in the levels of circulating testosterone [13]. Thus elderly men with coronary artery disease are likely to have levels of testosterone below the currently defined normal range, and, according to the results of the present study, will be significantly less sensitive to its vasodilatory properties than younger men. The resultant impairment of vasomotor function in the elderly male may be implicated in the presentation of coronary artery disease in the form of acute myocardial infarction, sudden cardiac death or symptomatic angina pectoris.

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