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# Intrinsic Responses of Rat Coronary Arteries In Vitro

## *Influence of Testosterone, Calcium, and Effective Transmural Pressure*

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**Administration of testosterone to men with angina has been shown to reduce myocardial ischemia. The mechanism of this effect is not clear but could be via an influence on coronary artery tone. We therefore employed an animal model to study the intrinsic responses of coronary arteries mounted in the wire myograph and evaluated the effect of testosterone on coronary artery tone in vitro. Intrinsic responses of the vessels and response to addition of testosterone were observed. Immediately after loading, vessels relaxed, an effect that was dependent on the baseline transmural pressure of vessels and was attenuated by pretreatment with *N*-nitro-L-arginine methyl ester. Subsequent contraction to a peak wall tension (intrinsic tone) was abolished by removal of extracellular calcium. Addition of testosterone produced a significant dose-dependent relaxation of intrinsic tone in all groups studied. The lowest concentration at which relaxation occurred was $10^{-6}$*M*. We conclude that rat coronary arteries exhibit calcium-dependent intrinsic responses and develop spontaneous tone. Furthermore, addition of testosterone reduces intrinsic coronary artery tone. These findings may have important implications for men with angina and low plasma testosterone levels.**

**Key Words:** Testosterone; coronary artery; intrinsic tone; calcium.

## Introduction

The worldwide gender difference in the incidence of cardiovascular diseases suggests a role for sex hormones in the etiology and progression of these conditions *(1)*. The role of estrogens in women has been widely investigated, although this currently remains unclear. The effects of androgens on the male cardiovascular system have received relatively little attention in comparison. Contrary to popular opinion, the available evidence suggests that androgens may be protective against atherosclerosis *(2–4)*. We have previously demonstrated that men with both stable coronary artery disease and acute myocardial infarction have lower plasma levels of testosterone than men with normal coronary arteries *(5,6)*. In addition, several studies have demonstrated an improvement in symptoms and objective measurements of ischemia in men with angina who were treated with testosterone, both acutely by iv injection *(7,8)* and long term by im injection or transdermal patch *(9,10)*. These findings point to a vasodilator effect of testosterone, which may relieve angina by reducing coronary artery intrinsic tone (the sustained state of contraction of a vessel under constant tension). This theory is supported by in vitro studies attempting to characterize the mechanism of action of testosterone on preconstricted vessels in various organ beds, which have suggested a calcium antagonist action of testosterone *(11–16)*. The low plasma testosterone levels seen in men with angina could therefore exacerbate patients' symptoms by impairment of coronary artery tone relaxation. However, the influence of testosterone has only been investigated in pharmacologically precontracted vessels, mostly in the wire myograph. Using such techniques, we previously studied potential mechanisms by which sex hormones have vasoactive effects and showed a gender-specific vasorelaxant effect of testosterone and 17β-estradiol *(17)*, which appears to be mediated by a calcium-antagonistic action *(18)*. The effect of testosterone on intrinsic coronary artery tone may be of greater clinical relevance, particularly in providing mechanistic insight into the apparent antianginal effect of testosterone. This has not been previously described. We therefore studied intrinsic responses of rat coronary arteries in the wire myograph and examined the influence of testosterone on the intrinsic tone developed by these vessels.

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## Results

There were no significant differences in the average age and weight of the animals studied in each group (Table 1).

### *Intrinsic Responses of Coronary Arteries*

Vessels in physiologic saline solution (+$Ca^{2+}$PSS) relaxed immediately after loading and then contracted. Wall tension rose to a peak, which preliminary investigations showed to be sustained over several hours (see Fig. 1). The intrinsic

**Table 1**
Characteristics of Animals and Vessels by Study Group[a]

| | Group | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | 100 mmHg | 80 mmHg | 60 mmHg | 40 mmHg | 20 mmHg | $PGF_{2\alpha}$ treated | Calcium free | L-NAME treated | L-NAME control |
| No. of animals | 6 | 6 | 7 | 7 | 7 | 6 | 4 | 6 | 7 |
| Weight (g)[b] | 392 (12.5) | 394 (15.1) | 355 (12.5) | 339 (12.8) | 385 (19.7) | 342 (29.4) | 403 (26.2) | 347 (30.3) | 342 (28.3) |
| No. of vessels | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 10 |

[a] Data are given as mean (SEM).
[b] $p = 0.31$ (ANOVA).

tone of each vessel was defined as the difference between the nadir and subsequent peak tensions recorded. The size of spontaneous contraction approached what had occurred in vessels treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1.87 ± 0.31 vs 2.13 ± 0.23 mN/mm; $p = 0.6$): 88% of pharmacologic precontraction.

### *Effect of Extracellular Calcium on Intrinsic Vessel Responses*

Vessels in calcium-free physiologic saline solution ($-Ca^{2+}$ PSS) also relaxed immediately after being loaded to baseline tension. The degree of relaxation from baseline was significantly different from that seen in vessels in $+Ca^{2+}$PSS (−1.15 ± 0.14 (24.6% of baseline tension) vs −1.50 ± 0.12 mN/mm (33.0%); $p < 0.0001$). The development of intrinsic tone was virtually abolished in vessels studied in calcium-free media (0.10 ± 0.07 vs 1.87 ± 0.31 mN/mm; $p < 0.0001$) (Fig. 1).

### *Effect of N-Nitro-L-Arginine Methyl Ester on Intrinsic Vessel Responses*

Relaxation of vessels was significantly reduced by pretreatment with *N*-nitro-L-arginine methyl ester (L-NAME) (16.9% vs 22.2% reduction from baseline tension; $p = 0.04$). However, development of intrinsic tone was not affected (1.34 ± 0.25 vs 1.25 ± 0.16 mN/mm; $p = 0.8$) (Fig. 1).

### *Effect of Baseline Tension on Intrinsic Vessel Responses*

The degree of initial relaxation varied significantly depending on the baseline transmural pressure, as did the time taken to reach the nadir (Fig. 2). Overall, there was no significant difference in the intrinsic tone developed by vessels in each group (Fig. 2). However, vessels loaded to 20 mmHg developed significantly less tone than those loaded at 100 mmHg ($p = 0.03$) and compared with all vessels loaded at >20 mmHg ($p = 0.008$). The time taken to develop intrinsic tone from onset to peak tension did not differ significantly among groups (Fig. 2).

### *Effect of Testosterone on Intrinsic Tone of Rat Coronary Arteries*

The exposure of vessels to testosterone produced a rapid (<5 min) dose-dependent relaxation of wall tension in all groups studied (Fig. 3). The minimum concentration of testosterone that induced significant relaxation was $10^{-6}M$ in vessels loaded at 100 mmHg ($p = 0.015$). Maximal relaxation was dependent on the baseline transmural pressure and was greatest in vessels loaded at higher baseline pressure.

## Discussion

These experiments show that under isometric tension in vitro, rat coronary arteries develop spontaneous tone, after initial relaxation. These phenomena have not previously been described in these vessels but have recently been studied in porcine pulmonary arteries. Using a pressure myograph, Liu and Sylvester *(19)* found that these vessels developed spontaneous tone when pressurized to 20 mmHg, with maximum effect after 135 min. The degree of tone developed was less than that seen in rat coronary arteries in the present study, being 35% of maximum possible vasoconstriction. The development of tone in pulmonary vessels also required the presence of extracellular calcium and was not altered by L-NAME, indomethacin (an inhibitor of prostacyclin synthesis), or blockade of endothelin-1. The investigators concluded that the spontaneous development of tone in isolated pulmonary arterioles resulted from spontaneous smooth muscle cell contraction, contributed to by decreasing activity of endothelium-derived relaxing factors other than nitric oxide (NO) and vasodilator prostaglandins or increasing activity of endothelium-derived contracting factors other than endothelin-1.

In the present study, the degree of relaxation was linearly related to the transmural pressure while, with tone, there appeared to be a threshold between 20 and 40 mmHg, above which the development of tone was more uniform. The initial relaxation of vessels was reduced by preincubation with L-NAME, suggesting that it is mediated, in part, by release of NO from the endothelium. Other vasorelaxant mediators may also be involved, such as endothelium-derived hyperpolarizing factor *(20)*. In addition, there may be a "rebound" effect of stretching the elastic connective tissue of the vessel wall.

The mechanism by which vessels develop spontaneous intrinsic tone is incompletely understood. A role for protein kinase C (PKC) has been implicated in the generation of

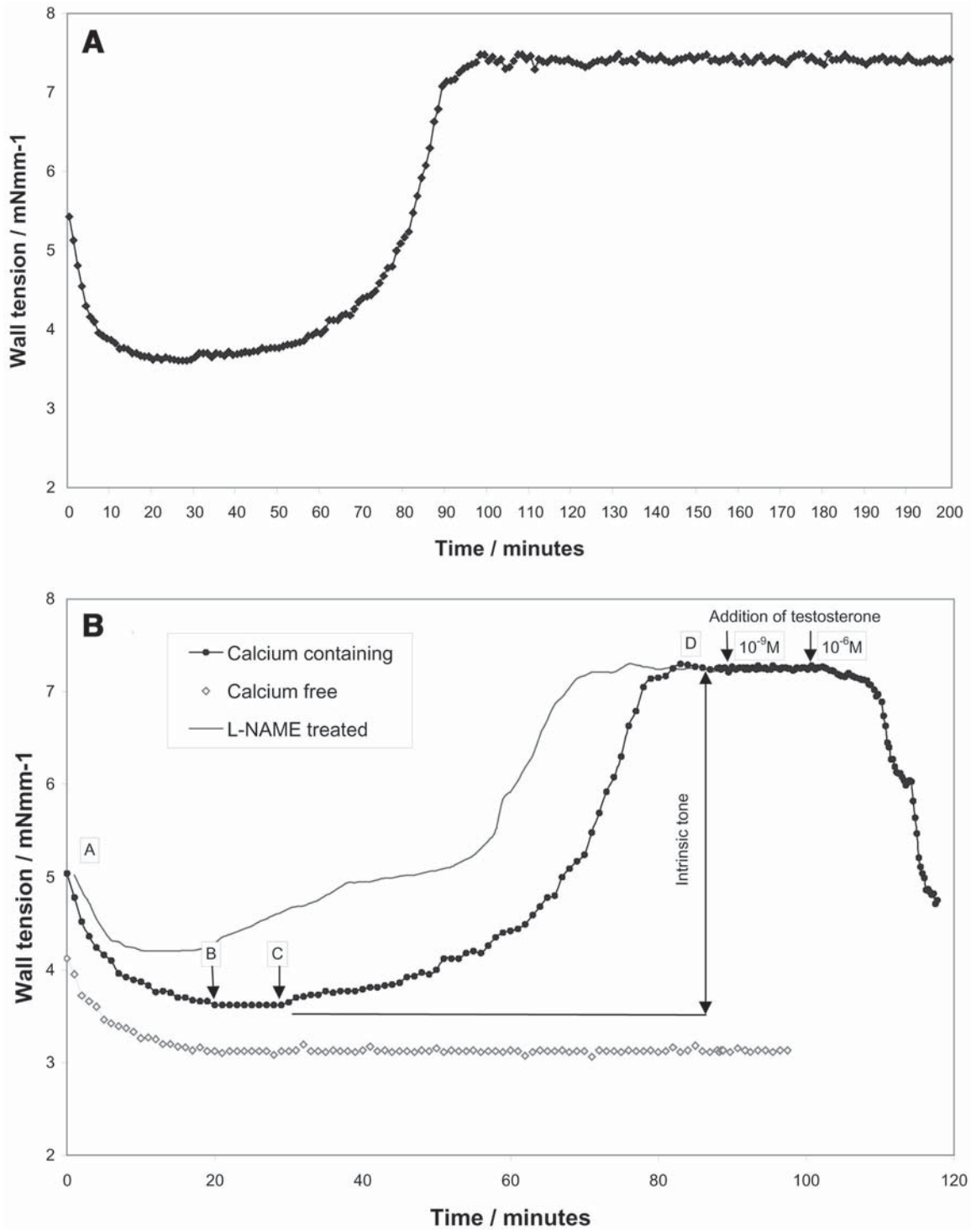


**Fig. 1.** Preliminary experiments demonstrated the pattern of the intrinsic response and the stability of the spontaneous tone (**A**). (**B**) Typical traces of vessels studied at 100 mmHg in calcium-containing, calcium-free, and L-NAME-treated PSS. A = loading wall tension, equivalent to a transmural pressure of 100 mmHg; B = nadir of wall tension; C = onset of spontaneous contraction (intrinsic tone); D = plateau at peak tension.

both intrinsic tone and the myogenic response by mobilization of intracellular calcium *(21,22)*. Utilization of extracellular calcium via voltage-dependent calcium channels may also play a role *(23)*. Miller et al. *(24)* have shown that intrinsic tone of human atrial coronary arterioles is reduced by both inhibition of voltage-dependent calcium channels by diltiazem and inhibition of PKC with calphostin C. Activation of PKC by phorbol 12-myristate 13-acetate led to enhancement of intrinsic tone. In the present study, removal of extracellular calcium virtually abolished the intrinsic tone, suggesting that, in rat coronary arteries, the influence of PKC is far less than that of voltage-dependent calcium channels.

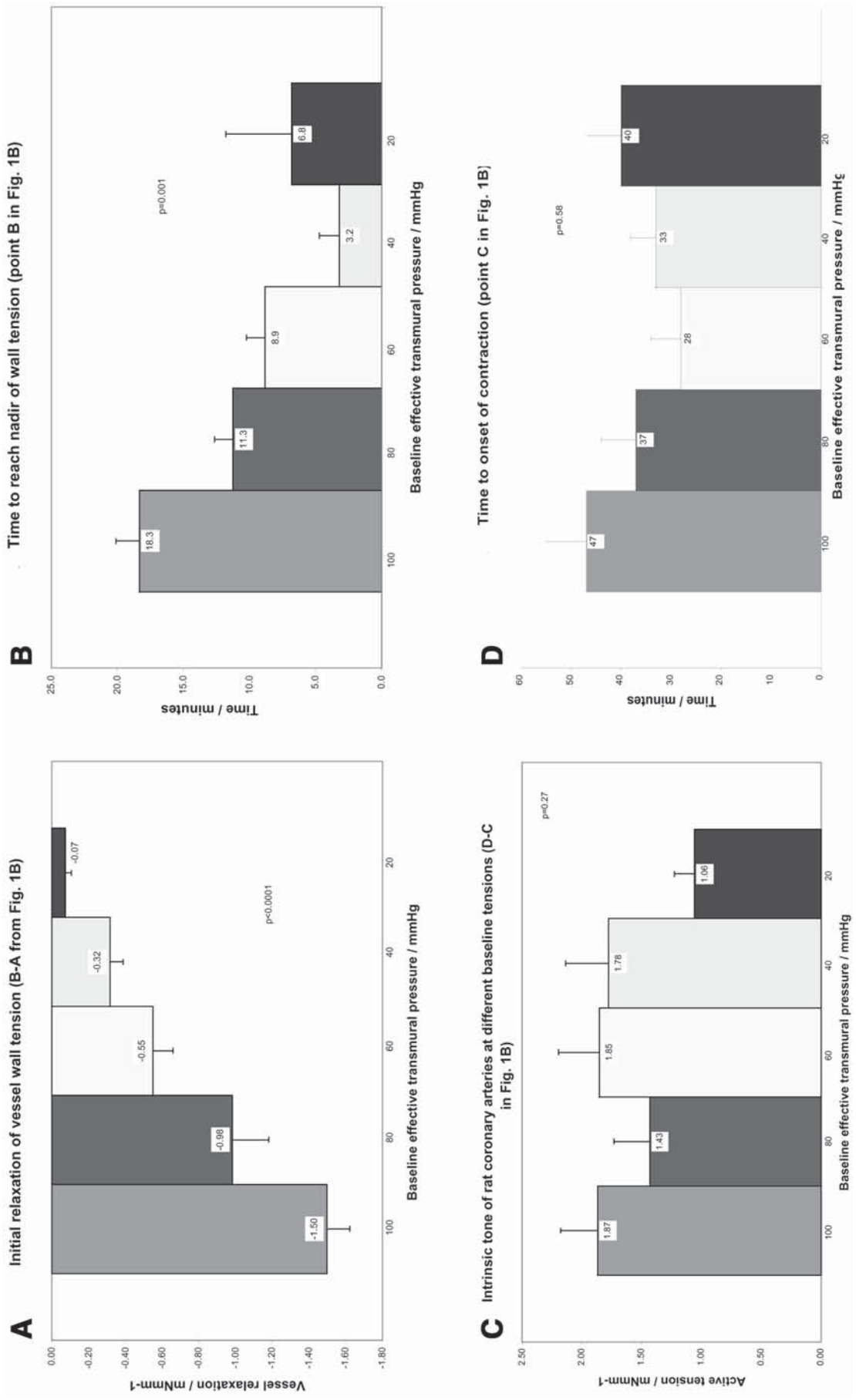


**Fig. 2.** Intrinsic responses of vessels, showing initial relaxation of vessels (**A**), time to reach nadir of wall tension (**B**), spontaneous tone of rat coronary arteries at different baseline tensions (**C**), and time to onset of contraction (**D**).

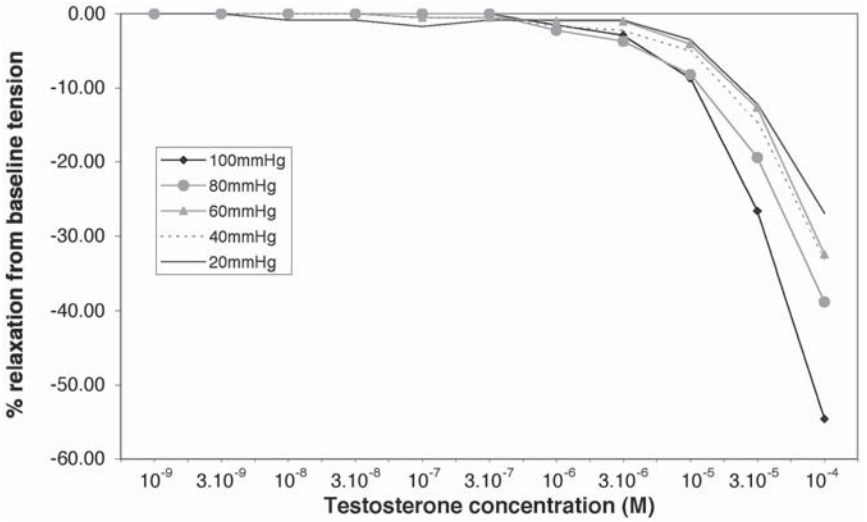


**Fig. 3.** Effect of testosterone at different wall tensions. Relaxation is shown as a percentage of the intrinsic tone.

The threshold effect observed for the development of tone is similar to that previously described in human atrial coronary arterioles studied on a pressure myograph and suggests the involvement of stretch receptors in the coronary artery wall *(24)*. Stretch-activated cation channels have been described in vascular smooth muscle cells *(25)*, which lead to elevation of intracellular calcium *(26)*, although their role in the development of intrinsic tone and in the myogenic response to stretch is not clear *(27)*.

In addition, our findings provide further evidence that testosterone acts as a coronary vasodilator and reduces calcium-dependent coronary artery intrinsic tone in vitro, suggesting that this may be how testosterone improves symptoms and objective measures of ischemia in men with angina. The novelty of these findings lies in the observation of these effects in vessels that had not been pharmacologically preconstricted but had been allowed to develop their own intrinsic tone.

The concentrations of testosterone that produced vasorelaxation in these experiments (micromolar range) were considerably higher than serum concentrations in men (nanomolar range), raising the question of whether these findings are of clinical relevance. It would appear that they are, given that administration of both high-dose and physiologic replacement dose testosterone to men with angina results in clinical improvement *(7–10)*. In addition, it is well recognized that discrepancies exist between the concentrations of agents required to produce effects in vitro and in vivo. In the case of testosterone, there are a number of possible reasons for this. There is evidence to suggest that testosterone may be actively absorbed in capillaries by the attachment of sex hormone binding globulin (SHBG) to specific receptor sites, with secondary active binding of testosterone to the SHBG molecule *(28)*. The concentration of active testosterone at the vessel membrane active site will therefore be many times higher than that estimated by serum samples. In addition, rats do not possess SHBG and may therefore require much higher doses to achieve the same effect as in animal models that do possess SHBG (i.e., humans). Thus, the high concentrations of testosterone employed in the present studies do not preclude an important physiologic and clinical role for testosterone in vivo.

The mechanism by which testosterone acts as a vasodilator is not yet clear but it appears to be independent of the endothelium *(11,18)*. The rapidity of onset of action of testosterone observed in the present study (within 5 min) also suggests that its effect is not mediated via the classic nuclear androgen receptor (AR), which would involve transcription of an effector protein, but via some other mechanism. This is supported by reports that the vasorelaxant effect of testosterone is unaffected by blockade of the nuclear AR by flutamide *(11,18)*. The rapidity of action also suggests that the vasorelaxant effect of testosterone is not mediated by aromatization to 17-estradiol, and this is supported by data showing that the effect of testosterone is unaffected in the presence of the aromatase inhibitor aminoglutethimide *(11)*. In addition, experimental data suggest that the mechanism of testosterone-induced vasodilatation involves both activation of potassium channels *(11,29,30)* and calcium channel antagonism *(15,17,18,31)*.

The results of our study add to the mounting evidence that testosterone plays an important role in the autoregulatory mechanisms controlling vascular tone in the coronary circulation, influencing not only agonist-induced coronary artery contraction but also intrinsic coronary artery tone. This may have important implications for men with angina and reduced plasma levels of testosterone.

## Materials and Methods

### *Chemicals and Solutions*

+$Ca^{2+}$PSS consisted of 120 m*M* NaCl, 4.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.17 m*M* $MgSO_4$, 25 m*M* $NaHCO_3$, 1.18 m*M* $KH_2PO_4$, 26.9 μ*M* EDTA and 5.5 m*M* glucose in distilled water. For –$Ca^{2+}$PSS, 1 m*M* EGTA was substituted for $CaCl_2$. For these experiments, a water-soluble preparation of testosterone was used. In this, each molecule of testosterone is encapsulated in a β-propyl-cyclodextrin carrier molecule complex, which releases the testosterone into solution on addition to water. Preliminary studies showed that the cyclodextrin carrier molecule had no vasoactive effect. All chemicals were obtained from Sigma, UK.

### *Preparation of Animals*

Male Wistar rats, 12 to 14 wk old, were sacrificed by cervical dislocation, according to UK Home Office guidelines. The heart was quickly removed and placed in cold +$Ca^{2+}$PSS. The left anterior descending and the right coronary arteries were carefully dissected free from surrounding tissue. Segments of vessel <2.5 mm long were isolated and mounted between the two jaws of the myograph (Cambustion, UK) on 40-μm stainless steel wires. The myograph chamber contained 5 mL of PSS heated to 37°C and bubbled continuously with 95% $O_2$/5% $CO_2$.

### *Development of Intrinsic Responses and Influence of Wall Tension*

The length-tension characteristics of each vessel were determined by the myograph computer. The myograph jaws were then separated, placing the vessel under tension determined by the computer to equate to a transmural pressure (the effective pressure) of either 20, 40, 60, 80, or 100 mmHg ($n$ = 12 in each group). Vessels were held under isometric conditions until the wall tension reached a plateau. Changes in wall tension were recorded. To compare spontaneous changes in vessel wall tension with pharmacologic precontraction, a set of 12 vessels was placed under tension equivalent to a transmural pressure of 100 mmHg. $PGF_{2\alpha}$ was added to the organ bath in a concentration of $10^{-4}$*M* and changes in wall tension were recorded.

### *Influence of Calcium on Intrinsic Vessel Responses*

A further set of 12 vessels was placed under tension equivalent to a transmural pressure of 100 mmHg in –$Ca^{2+}$PSS. Vessels were held in isometric conditions, and changes in wall tension were noted and compared with those occurring in vessels under similar tension in +$Ca^{2+}$PSS.

### *Influence of Endothelium on Intrinsic Vessel Responses*

Initial studies revealed that vessels partially relaxed, immediately after loading to baseline tension. The influence of NO release on initial relaxation and on the subsequent development of intrinsic tone was studied by preincubating of vessels for 30 min with $10^{-5}$*M* L-NAME ($n$ = 10) or vehicle control ($n$ = 10) prior to loading to 100 mmHg. Previous studies have shown that this concentration is sufficient to block NO release in this study preparation *(32)*. Vessels were held in isometric conditions, and changes in wall tension were noted and compared between the two groups.

### *Effect of Testosterone on Intrinsic Coronary Artery Tone*

After the wall tension of vessels in +$Ca^{2+}$PSS had reached a plateau, testosterone was added cumulatively to the organ bath to produce stepwise increases in bath concentration of testosterone, ranging from $10^{-9}$*M* to $10^{-4}$*M*. Subsequent changes in wall tension were noted.

### *Statistical Analyses*

Data are presented as mean ± SEM unless otherwise stated. Mean values were compared among all groups by analysis of variance (ANOVA). Two group comparisons were performed using student's *t*-test for parametric data and Mann-Whitney *U* test for nonparametric data. Changes in wall tension following administration of testosterone were analyzed by analysis of covariance, with baseline tension as covariate, or by the paired *t*-test and are presented as a percentage of the intrinsic tone. A $p$ value <0.05 was considered significant.

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