EXPERIMENTAL STUDY

Altered circulating hormone levels, endothelial function and vascular reactivity in the testicular feminised mouse

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Abstract

Objective: Testicular feminised (Tfm) mice express a non-functional androgen receptor, and also have reduced levels of circulating testosterone. Recent studies support a cardio-protective role for testosterone since it elicits systemic and pulmonary vasodilatation. The aim of the present study was to determine whether androgen insensitivity and hypotestosteronaemia in the Tfm mouse are associated with abnormal vascular reactivity or hormone status.

Methods: Adult male Tfm and littermate control mice were killed and the blood collected. Femoral (diameter range = 183–508 μm) and pulmonary (diameter range = 320–816 μm) arteries were dissected and loaded in either a wire or pressure myograph, at 100 mmHg or 17.5 mmHg respectively. Pharmacological assessment of the vasoreactivity to potassium chloride (KCl, 80 mmol/l) and either noradrenaline (NA, 1 nmol/l–100 $\mu\text{mol/l}$) and acetylcholine (ACh, 0.1–100 $\mu\text{mol/l}$) or testosterone (1 nmol/l–100 $\mu\text{mol/l}$) was then made.

Results: Tfm mice had reduced levels of testosterone (1.8 ± 0.3 nmol/l) compared with controls (9.3 ± 2.0 nmol/l, $P < 0.001$) and elevated levels of cholesterol (3.6 ± 0.1 mmol/l) compared with controls (3.2 ± 0.1 mmol/l, $P < 0.05$). Femoral arteries from Tfm mice exhibited reduced vasoconstriction to 80 mmol/l KCl (3.27 ± 0.23 mN/mm) compared with vessels from controls (4.44 ± 0.41 mN/mm, $P < 0.05$), and reduced endothelial-dependent vasodilatation to 0.1–100 $\mu\text{mol/l}$ ACh ($23.3 \pm 3.6\%$ relaxation) compared with vessels from controls ($41.6 \pm 5.4\%$ relaxation, $P < 0.05$). Vasoconstriction to NA (1 nmol/l–100 $\mu\text{mol/l}$) and vasodilatation to testosterone were unaffected.

Conclusions: Androgen receptor deficiency and hypotestosteronaemia in the Tfm mouse reduced endothelial function and impaired voltage-operated calcium channel activity, which may pre-dispose to cardiovascular disease. Testosterone-induced vasodilatation was unaffected, demonstrating no involvement of the androgen receptor in this response.

European Journal of Endocrinology 148 111–120

Introduction

The testicular feminised (Tfm) mouse is the murine equivalent of the human condition *complete androgen insensitivity syndrome* (CAIS). Affected animals exhibit an X-linked, single base-pair deletion in the gene encoding the androgen receptor (1). Gene transcription consequently produces a truncated form of the receptor protein, which has a conformational change in the androgen binding site, preventing testosterone binding (2). As such these animals are rendered completely androgen insensitive and therefore phenotypically female. Because activation of the pituitary androgen receptors involved in the negative long loop feedback cannot occur, it has been hypothesised that these animals should have elevated circulating levels of

luteinising hormone (LH) and therefore testosterone. However, whilst LH levels are elevated, circulating levels of testosterone are reduced in the Tfm mouse (3). This is due to a deficiency of the enzyme 17α hydroxylase in the Leydig cells of the testis (3, 4), a key constituent of the steroidogenesis pathway, the activity of which is postulated to be reduced both by the intra-abdominal location of the testes, and by the absence of androgen action *in utero* (3). Consequently, affected animals not only have a non-functional androgen receptor but also have a reduced circulating testosterone profile.

The role that sex hormones play in heart disease is an area that has recently received growing consideration by cardiovascular researchers. Even after correcting for differences in risk factors between the two sexes,

men are twice as likely as women to die from coronary artery disease (CAD) (5, 6). This epidemiological evidence coupled with data suggesting that hormone replacement therapy in women is protective, and that anabolic steroid abuse can cause heart disease (reviewed in refs 7 and 8), has led to the assumption that androgens are bad for the heart. However, several studies over the last decade now suggest the opposite – that testosterone is beneficial to the male cardiovascular system, and that a relative deficiency in circulating levels may lead to detrimental effects. Indeed, hypotestosteronaemia is associated with numerous risk factors for CAD including hypertension, hyperinsulinaemia, diabetes, obesity, age and adverse thrombotic profile (reviewed in ref. 9), and testosterone levels have been reported to be inversely proportional to atherosclerotic risk (10–14). Recent work by our group has demonstrated that men with significant CAD (greater than 75% stenosis on angiography) have significantly lower levels of bioavailable testosterone than a group of age- and weight-matched men with normal coronary arteries (15). This is in agreement with the findings of numerous cross-sectional case control studies which also report hypotestosteronaemia in patients with heart disease (reviewed in ref. 16). Testosterone therapy has been shown to improve myocardial ischaemia in patients with CAD, whether administered orally (17), via intra-muscular injection (18) or by transdermal patch (19). Direct intra-coronary infusion of testosterone in men with CAD induces coronary vasodilation (20) and testosterone-induced vasodilatation has also been demonstrated in animals models, encompassing a variety of vascular beds, from an assortment of species (21–27).

Clearly, evidence supports a cardio-protective role for testosterone. However, it remains unknown whether the reduction in the circulating testosterone profile seen in male patients with CAD is a causative factor in the development of this condition, or merely a consequence of a chronic disease state. The aim of the present study was to utilise the Tfm mouse model, to determine whether any alterations in vascular reactivity and/or vessel compliance are associated with the lack of a functional androgen receptor and reduced circulating testosterone profile, seen in this model. Such alterations may manifest as cardiovascular disease in later life, and therefore give an indication of whether hypotestosteronaemia is a cause or consequence of CAD.

Materials and methods

Tfm mouse colony

A breeding colony of Tfm mice (strain C57BL/6 J-A) was re-derived from frozen embryos obtained from the MRC mouse genome project, Harwell, Oxfordshire,

Figure 1 The two breeding schemes (1 and 2) utilised for the generation of Tfm mice ($X^{Tfm}Y$ – agouti) from carrier females ($X^{Tfm}X^{Blo}$ – light patches and $X^{Tfm}X^{Ta33H}$ – tabby stripes) and non-carrier males (X^{Ta33H}/Y – dark and X^{Blo}/Y – light). Non-carrier females are also depicted ($X^{Ta33H}X^{Blo}$ – stripes and patches).

UK. The genotype of the animals was determined via an inherent coat colour marker, and equal numbers of the two breeding schemes depicted in Fig. 1 were maintained in sterile barrier conditions at The University of Sheffield Field Laboratories, Western Bank, Sheffield. Non-carrier females ($X^{Ta33H}X^{Blo}$) and carrier females ($X^{Tfm}X^{Ta33H}$ or $X^{Tfm}X^{Blo}$) not required for breeding were killed via a UK Home Office-approved Schedule 1 method, once weaned. Male offspring (X^{Ta33H}/Y or X^{Blo}/Y) that were not required for re-stocking the breeding colony, and Tfm offspring ($X^{Tfm}Y$) were transferred at 6 weeks of age to the local field laboratories based at the Royal Hallamshire Hospital, Sheffield.

Assessment of plasma hormone levels

Tfm mice ($X^{Tfm}Y$) and male littermate controls (X^{Ta33H}/Y and X^{Blo}/Y) of a similar age as those used in subsequent experiments (Tfm, $n = 46$, mean age = 75 ± 1 days; controls, $n = 40$, mean age = 74 ± 1 days) or between 3 and 8 months old (Tfm, $n = 42$, mean age = 140 ± 7 days; controls $n = 51$, mean age = 151 ± 7 days) were killed via cervical dislocation as approved by the UK Home Office. A mid-line sternotomy was performed, the rib-cage opened, and the blood removed from the chest cavity following severance of the thoracic aorta, using a sterile 1 ml pipette. The blood was then transferred to a 1.5 ml Eppendorf tube, placed on ice and taken immediately to the research laboratory. The blood was spun in a centrifuge for 10 min at 3000 r.p.m., the plasma collected and transferred to a fresh 1.5 ml Eppendorf tube and stored at -20°C for later analysis. Total testosterone levels were measured using commercially available ELISA kits (Immuno Diagnostic Systems Ltd, Boldon, Tyne and Wear, UK). Total cholesterol, progesterone

and cortisol levels were analysed by the Department of Clinical Chemistry, Royal Hallamshire Hospital, Sheffield, using in-house ELISA. All ELISA analysis was undertaken using mouse antibodies.

Assessment of vascular reactivity of testosterone by wire myography

Femoral arteries ($n = 48$, mean internal diameter = $322 \pm 9 \mu\text{m}$) from Tfm ($n = 8$, mean age = 72 ± 2 days) and littermate control mice ($n = 8$, mean age = 72 ± 2 days), and pulmonary arteries ($n = 46$, mean internal diameter = $512 \pm 14 \mu\text{m}$) from Tfm ($n = 7$, mean age = 72 ± 1 days) and littermate control mice ($n = 7$, mean age = 74 ± 1 days), were used in this series of experiments. Between three and four femoral and pulmonary vessels were dissected initially from each animal, although four pulmonary arteries and three femoral arteries were later discarded due to either having a damaged endothelial or smooth muscle cell layer (see below).

The technique of wire myography is commonly utilised for vascular reactivity studies and has been described in detail elsewhere (28). Briefly, vessels were mounted on two $40 \mu\text{m}$ stainless steel wires in the jaws of an automated wire myograph (Cambustion Ltd, Cambridge, Cambridgeshire, UK), the myograph bath filled with physiological saline solution (PSS) heated to 37°C and bubbled with 95% $\text{O}_2/5\%$ CO_2 to maintain a physiological pH of 7.4. Length-tension characteristics were obtained via the myograph software (Cambustion Ltd) and on the basis of these, femoral arteries were loaded to a tension equivalent to an *in vivo* pressure of 100 mmHg, and pulmonary arteries were loaded to a tension equivalent to an *in vivo* pressure of 17.5 mmHg. The vessels were then left to equilibrate under these conditions for 1 h, after which they were exposed three times to 80 mmol/l potassium chloride (KCl), to ensure that the vessels were viable and that responses to applied agonists were reproducible. Vessels were then washed and exposed to noradrenaline (NA; 10 $\mu\text{mol/l}$), and once a maximal contraction had been produced, acetylcholine (ACh; 10 $\mu\text{mol/l}$) was added to confirm the presence of an intact endothelial cell layer. The vessels were then washed, re-exposed to NA (10 $\mu\text{mol/l}$) and once a maximal contraction had been achieved, cumulative additions of either testosterone (0.1–100 $\mu\text{mol/l}$) or an equivalent volume of ethanol vehicle were made, and changes in tension recorded. The final bath concentration of ethanol was $<0.3\%$.

Assessment of vascular reactivity of testosterone by pressure myography

Femoral arteries ($n = 24$, mean external diameter = $426 \pm 11 \mu\text{m}$) were dissected carefully from Tfm ($n = 12$, mean age = 74 ± 2 days) and littermate control

mice ($n = 12$, mean age = 78 ± 2 days) (one vessel per animal), for study by pressure myography. Again, the technique of pressure myography has been described in detail elsewhere (29). Briefly, vessels were mounted on two glass pipettes in a pressure myograph system (Living Systems, Burlington VT, USA), and held in place with three strands of synthetic thread at either end. The myograph bath was filled with PSS heated to 37°C and bubbled with 95% $\text{O}_2/5\%$ CO_2 to maintain a physiological pH of 7.4. The end of the pipette distal to the vessel was sealed, and PSS pumped slowly into the vessel using a pressure servo, via capillary tubing attached to the proximal end of the vessel. An in-line pressure transducer was located in the circuit proximal to the vessel to enable measurement of the pressure within the system. The vessel was then slowly pressurised in 10 mmHg gradations to a resting pressure of 100 mmHg. A feedback loop to the pressure servo ensured that the vessel was held at this constant resting pressure. External diameter was monitored continuously using a Video Dimension Analyser (Living Systems). Signals from the pressure transducers and Video Dimension Analyser were digitised and stored on a computer loaded with WINDAQ data acquisition system software (Dataq Instruments, Akron, OH, USA). The vessels were left to equilibrate under these conditions for 1 h, and were then exposed three times to 80 mmol/l KCl, to ensure that the vessels were viable and that responses to applied agonists were reproducible. Vessels were then washed and exposed to NA (1 $\mu\text{mol/l}$), and once a maximal contraction had been produced, ACh (10 $\mu\text{mol/l}$) was added to confirm the presence of an intact endothelial cell layer. The vessels were then washed, re-exposed to NA (1 $\mu\text{mol/l}$) and once a maximal contraction had been produced, cumulative additions of either testosterone (1 nmol/l–100 $\mu\text{mol/l}$) or an equivalent volume of ethanol vehicle were made, and changes in external vessel diameter recorded. The final bath concentration of ethanol was $<0.5\%$.

Assessment of changes in agonist-induced responses and endothelial function

Femoral arteries ($n = 40$, mean internal diameter = $332 \pm 9 \mu\text{m}$) from Tfm ($n = 7$, mean age = 71 ± 2 days) and littermate control mice ($n = 7$, mean age = 72 ± 2 days) were used in this series of experiments. Three femoral arteries were initially dissected from each animal, although two were later discarded due to having a damaged smooth muscle cell layer. Vessels were mounted on two $40 \mu\text{m}$ stainless steel wires in the jaws of an automated wire myograph (Cambustion Ltd) as described above. After the 1 h equilibration period the vessels were exposed three times to 80 mmol/l KCl, to ensure that the vessels were viable and that responses to applied agonists were reproducible.

Vessels were then washed and exposed to NA (1 nmol/l–100 μ mol/l), and once a maximal contraction had been produced, ACh (0.1–100 μ mol/l) was added and changes in tension recorded.

Assessment of changes in vascular compliance

Femoral arteries ($n = 37$, mean internal diameter = $326 \pm 10 \mu\text{m}$) were dissected carefully from Tfm ($n = 7$, mean age = 71 ± 2 days) and littermate control mice ($n = 7$, mean age = 72 ± 2 days) for the assessment of vessel compliance. Three vessels were dissected per animal, but five vessels were not included in the analysis (four from Tfm and one from control animals) on this basis that they had received excessive handling, and in the absence of pharmacological evidence we could not be sure that damage to the smooth muscle cell layer had not occurred.

Vessels were mounted on two 40 μm stainless steel wires in the jaws of an automated wire myograph (Cambustion Ltd) as described above. Diameter–tension curves were then obtained for each vessel, as previously described (30). Starting at a diameter that yielded no tension on the vessel, the myograph jaws were separated at a rate of 50 $\mu\text{m/s}$ until the force exerted on each vessel 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 two diameter tension curves were stored within the myograph software (Cambustion Ltd) and the mean of the two curves plotted. The change in diameter divided by the change in tension ($\Delta x/\Delta y$) was then calculated as a measure of dynamic vessel compliance ($\mu\text{m/mN}$). This was measured over the diameter range $\pm 50 \mu\text{m}$ of the diameter at which each vessel possessed a tension equivalent to 100 mmHg. This diameter range was selected as it

was representative of the *in vivo* variability in vessel diameter.

Solutions and drugs

PSS consisted of 120 mmol/l NaCl, 4.7 mmol/l KCl, 1.17 mmol/l MgSO_4 , 25 mmol/l NaHCO_3 , 1.18 mmol/l KH_2PO_4 , 5.5 mmol/l glucose, 2.5 mmol/l CaCl_2 and 26.9 $\mu\text{mol/l}$ EDTA dissolved in distilled water. All PSS reagents were obtained from Sigma UK Ltd. NA, ACh and KCl were all obtained from Sigma UK Ltd and dissolved in distilled water to the required concentration. Testosterone was also obtained from Sigma UK Ltd but was dissolved in ethanol.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical analysis was made by Student's paired or unpaired *t*-test for parametric data, and by Mann–Whitney U test for non-parametric data. In all cases $P < 0.05$ was considered to be statistically significant.

Results

Plasma hormone levels

The hormone levels from the Tfm (X^{TfmY}) and male littermate control mice (X^{Ta33HY} and X^{BloY}) are shown in Table 1. The intra- and interassay coefficients of variance were $< 5\%$ in all cases. Because the hormone measurement assay consumed a significant volume of blood from each sample, in some cases insufficient sample remained for some of the subsequent assays. As such, from the 179 plasma samples collected, 179 values of total testosterone, 117 values of total cholesterol, 58 values of total progesterone and 48 values of total cortisol were obtained.

Table 1 Mean (S.E.M.) plasma hormone levels from adult Tfm and littermate control mice.

	X^{TfmY}	XY	X^{TfmY}	XY
Age (days)	75 (1)	74 (1)	140 (7)	151 (7)
<i>n</i>	46	40	42	51
Total testosterone (nM)	1.8 (0.3)	9.3 (2.0) [†]	1.4 (0.1)	16.5 (2.9) [†]
<i>n</i>	46	40	42	51
Total cholesterol (mM)	3.6 (0.1)	3.2 (0.1)*	3.6 (0.3)	2.8 (0.1)**
<i>n</i>	39	35	22	21
Total progesterone (nM)	4.0 (0.4)	4.9 (0.6)	5.9 (0.5)	4.7 (0.5)
<i>n</i>	7	26	8	17
Total cortisol (nM)	28.1 (0.5)	30.8 (2.2)	28.0 (1.0)	40.5 (4.5)
<i>n</i>	21	23	2	2

* $P < 0.05$, ** $P < 0.01$, [†] $P < 0.001$ compared with age-matched Tfm mice via Student's unpaired *t*-test.

Table 2 Vessel characteristics and baseline vasoreactivity to KCl, NA and ACh for the pulmonary and femoral arteries isolated from Tfm and littermate control mice, utilised for the study of the vascular reactivity of testosterone. Results are expressed as means $\pm$ S.E.M.

	XY Pulmonary arteries	Tfm Pulmonary arteries	XY Femoral arteries (wire myography)	Tfm Femoral arteries (wire myography)	XY Femoral arteries (pressure studies)	Tfm Femoral arteries (pressure studies)
<i>n</i>	23	23	25	23	12	12
Diameter (μ m)	525 $\pm$ 20	498 $\pm$ 21	315 $\pm$ 13	330 $\pm$ 13	420 $\pm$ 17	430 $\pm$ 16
Contraction to 80 mM KCl	1.59 $\pm$ 0.13 mN/mm	1.65 $\pm$ 0.20 mN/mm	4.67 $\pm$ 0.40 mN/mm	3.37 $\pm$ 0.24** mN/mm	- 173 $\pm$ 14 $\Delta\mu$ m	- 128 $\pm$ 11 $\Delta\mu$ m
Contraction to 1 μ M NA	-	-	-	-	- 152 $\pm$ 14 $\Delta\mu$ m	- 176 $\pm$ 14 $\Delta\mu$ m
Contraction to 10 μ M NA	0.97 $\pm$ 0.10 mN/mm	1.13 $\pm$ 0.07 mN/mm	4.67 $\pm$ 0.51 mN/mm	4.16 $\pm$ 0.34 mN/mm	-	-
Dilatation to 10 μ M ACh	- 0.30 $\pm$ 0.06 mN/mm	- 0.25 $\pm$ 0.03 mN/mm	- 2.14 $\pm$ 0.39 mN/mm	- 1.08 $\pm$ 0.23* mN/mm	50 $\pm$ 11 $\Delta\mu$ m	53 $\pm$ 13 $\Delta\mu$ m

* $P < 0.05$ and ** $P < 0.01$ compared with similar vessels from control animals via Student's unpaired *t*-test.

Tfm mice had a total testosterone level significantly lower than the littermate controls, and also exhibited an elevated level of total cholesterol. This was consistent whether analysis was made in animals of experimental age or animals of mature adult age. No significant alterations were seen in the circulating plasma levels of progesterone or cortisol between Tfm and littermate controls.

Vascular reactivity of testosterone – wire myography

The vessel characteristics and vasoreactivity of the pulmonary arteries and femoral arteries utilised in this series of experiments are shown in Table 2.

The diameter and baseline responsiveness to KCl (80 mmol/l), NA (10 μ mol/l) and ACh (10 μ mol/l) of the pulmonary arteries from Tfm and littermate control mice were similar in all cases (Table 2). Vasodilatation to testosterone was also similar in pulmonary arteries from Tfm and littermate control mice (Fig. 2A). Significant dilatation to testosterone (compared with ethanol vehicle) was seen at 10, 30 and 100 μ mol/l, with approximately 100% reversal of the pre-contractile tone induced by NA (10 μ mol/l) occurring at 100 μ mol/l (Fig. 2A). Significantly greater dilatation to testosterone was seen at a concentration of 30 μ mol/l in vessels from littermate control animals (Fig. 2A).

The diameter and baseline responsiveness to NA (10 μ mol/l) was also similar in femoral arteries from Tfm and littermate control mice (Table 2). However the contraction induced by KCl (80 mmol/l) and the dilatation to ACh (10 μ mol/l) was reduced in femoral arteries from Tfm mice (Table 2). As with the pulmonary arteries, the vasodilatory efficacy of testosterone was not significantly altered in femoral arteries

from Tfm mice (Fig. 2B), although the potency of testosterone was enhanced compared with pulmonary arteries. Significant dilatation to testosterone (compared with ethanol vehicle) was seen at 1, 3, 10, 30 and 100 μ mol/l in femoral arteries from littermate controls, and at 3, 10, 30 and 100 μ mol/l in femoral arteries from Tfm animals (Fig. 2B). Again, approximately 100% reversal of the pre-contractile tone induced by NA (10 μ mol/l) occurred at 100 μ mol/l (Fig. 2B). Responses to testosterone in femoral arteries from Tfm and littermate control mice did not differ significantly at any concentration tested.

Vascular reactivity of testosterone – pressure myography

The diameter and baseline responsiveness to KCl (80 mmol/l), NA (1 μ mol/l) and ACh (10 μ mol/l) were similar in femoral arteries from Tfm and littermate control mice utilised in this series of experiments (Table 2). As with the femoral arteries studied by wire myography, the vasodilatory efficacy of testosterone was not significantly altered in femoral arteries from Tfm mice (Fig. 2C). Significant dilatation to testosterone (compared with ethanol vehicle) was seen at 3, 10, 30 and 100 μ mol/l in femoral arteries from both Tfm and littermate control mice (Fig. 2C). Only approximately 70% reversal of the pre-contractile tone induced by NA (1 μ mol/l) was seen at 100 μ mol/l testosterone (Fig. 2C). Responses to testosterone in femoral arteries from Tfm and littermate control mice did not differ significantly at any concentration tested.

Vascular reactivity of NA and ACh

The femoral arteries harvested from Tfm and littermate control mice were of similar diameter: 334 $\pm$ 14 μ m

Figure 2 The concentration–response curve to testosterone compared with ethanol vehicle in isolated pulmonary arteries studied via wire myography (A), in isolated femoral arteries studied via wire myography (B) and in isolated femoral arteries studied via pressure myography (C). ●, Response to testosterone in vessels harvested from Tfm mice. ■, Response to testosterone in vessels harvested from control mice. ○, Response to ethanol vehicle in vessels harvested from Tfm mice. □, Response to ethanol vehicle in vessels harvested from control mice. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the respective vehicle-exposed control vessels via Mann–Whitney U test. Results are expressed as means $\pm$ S.E.M.

($n = 19$) and $312 \pm 13 \mu\text{m}$ ($n = 21$) respectively, but their vasoreactivity was significantly altered. Mean contraction to KCl (80 mmol/l) was reduced in vessels from Tfm mice: 3.27 ± 0.23 mN/mm compared with 4.44 ± 0.41 mN/mm in vessels from littermate controls ($P < 0.05$, Student's unpaired t -test). Endothelial-dependent vasodilatation to ACh (0.1–100 $\mu\text{mol/l}$) was also significantly reduced in vessels from Tfm mice: $E_{\text{max}} = 23.3 \pm 3.6\%$ relaxation compared with $41.6 \pm 5.4\%$ relaxation in vessels from littermate control mice ($P < 0.05$, Student's unpaired t -test) (Fig. 3). Significantly lower vasodilatation to ACh was seen at concentrations of 0.1, 0.3, 1 and 30 $\mu\text{mol/l}$ (Fig. 3). The contractile response to NA (1 nmol/l–100 $\mu\text{mol/l}$) was similar in vessels from Tfm and littermate control mice (Fig. 4).

Vascular compliance

The femoral arteries harvested from Tfm and littermate control mice were of similar diameter: $342 \pm 14 \mu\text{m}$

Figure 3 The concentration–response curve to ACh in isolated femoral arteries harvested from Tfm (●) and littermate control mice (○). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ via Student's unpaired t -test. Results are expressed as means $\pm$ S.E.M.

Figure 4 The concentration–response curve to NA in isolated femoral arteries harvested from Tfm (●) and littermate control mice (○). Results are expressed as means $\pm$ S.E.M.

($n = 17$) and $312 \pm 13 \mu\text{m}$ ($n = 20$) respectively. The compliance of the vessels from the Tfm mice was lower than the compliance of the vessels from littermate control mice: $5.09 \pm 0.36 \mu\text{m/mN}$ and $5.98 \pm 0.37 \mu\text{m/mN}$ respectively, although this failed to reach statistical significance ($P = 0.1$, Student's unpaired *t*-test).

Discussion

The main findings of the present study were that androgen receptor deficient Tfm mice had significantly altered circulating plasma levels of both testosterone and cholesterol, and that these alterations were associated with impaired endothelial-dependent vasodilatation and reduced vascular reactivity to KCl. The vasodilatory action of testosterone was not altered significantly.

Tfm mice had an approximately tenfold lower circulating level of total testosterone compared with littermate control animals, a deficiency that was maintained across all the age ranges of animals tested (8 weeks to 8 months old). The fact that Tfm mice have a reduced circulating testosterone profile has previously been reported (3) and is attributed to a deficiency of the enzyme 17α hydroxylase (3, 4), which is a key constituent of the steroidogenesis pathway whereby sex steroids are synthesised from cholesterol in the gonads. The effect of this enzyme deficiency on the circulating levels of other steroid hormones has not previously been reported. In the present study we have demonstrated that Tfm mice also have an elevated level of circulating plasma cholesterol compared with littermate controls (Table 1), and although this rise was modest, it reached statistical significance in all groups tested. Hence the present study has demonstrated an elevation in the levels of the initial substrate of the steroidogenesis pathway, and a reduction in the levels of the end product, consistent with 17α hydroxylase deficiency. However the levels of progesterone, another substrate for 17α hydroxylase, were not significantly altered (Table 1), although this may be a consequence of the relatively low numbers of samples analysed from the Tfm group (15 compared with 43) (Table 1). The levels of cortisol, another product downstream of 17α hydroxylase, were reduced in Tfm animals (Table 1), but this also did not reach statistical significance for the number of samples analysed.

We then went on to determine whether these changes in the circulating hormone profile had any effect upon vascular reactivity, initially responsiveness to testosterone. Previous work has demonstrated that testosterone elicits vasodilatation via a mechanism that is postulated to be independent of the nuclear androgen receptor due to its rapid onset and insensitivity to the androgen receptor blocker flutamide (21, 25, 26). Furthermore, the vasodilatory response is preserved

when testosterone is covalently bound to bovine serum albumin, which prevents the entry of testosterone into the smooth muscle cell, thus preventing interaction with the nuclear androgen receptor (31). Whilst this strongly suggests that testosterone does not elicit vasodilation via interaction with this nuclear androgen receptor, it does not rule out activation of a membrane-bound androgen receptor, as has been proposed in other cells (32–34). Indeed, we have been able to demonstrate the presence of a membranous testosterone binding site in cultured arterial smooth muscle cells (35) and this may be a receptor governing testosterone-induced vasodilatation. Preliminary experiments demonstrated that the vasodilatory activity of testosterone was unaffected in isolated thoracic aortae from Tfm mice (36). In the present study we wanted to determine whether the vasodilatory action of testosterone in other vascular beds was also independent of this genomic signalling pathway, and if the potency of testosterone could be enhanced using a more physiological technique. Consequently, we studied the vasodilatory action of testosterone in isolated pulmonary and femoral arteries using both wire and pressure myography. Due to the branched nature of the pulmonary vascular bed, pulmonary arteries could not be pressurised in this system, and consequently these experiments were restricted to femoral arteries.

Testosterone-induced vasodilatation in both these vascular beds and preparations was unaffected by the absence of a functional androgen receptor, and by the observed testosterone deficiency. These data further substantiate the hypothesis that testosterone-induced vasodilatation does not involve activation of the androgen receptor, and also suggests that the membranous testosterone binding site previously identified by our group and hypothesised to mediate the vasodilatory activity of testosterone, is not an androgen receptor. Furthermore, because the vasodilatory capacity of testosterone is not potentiated in vessels from testosterone-deficient Tfm mice, it would also suggest that this binding site may not be a testosterone-specific receptor, since upregulation of the 'receptor' has clearly not occurred. This adds further weight to the argument that testosterone may be capable of regulating vascular tone via interaction with an ion channel in the smooth muscle cell membrane, as has been suggested previously by ourselves and others (21, 23–27).

The concentration at which testosterone produced vasodilatation remained in the pharmacological, rather than physiological, range, despite using the more physiological technique of pressure myography (Fig. 2B,C). However, such a difference between *in vitro* experimentation and the *in vivo* situation is common. For example, cromakalin, a potassium channel opener, reduces systolic arterial pressure and systemic vascular resistance *in vivo* at concentrations of $2\text{--}3 \times 10^{-8} \text{ mol/l}$. *In vitro*, however, vasodilatory effects are only seen at $10^{-6}\text{--}10^{-5} \text{ mol/l}$, a similar discrepancy

to the present situation (circulating total testosterone levels being approximately 10^{-8} mol/l – Table 1). The necessity of a high concentration of testosterone to induce vasodilatation raises the issue of whether cross-reactivity at oestrogen receptors or conversion to 17β oestradiol via the enzyme aromatase occurs. However this is unlikely for a number of reasons. Firstly, a number of studies have directly investigated the involvement of oestrogens in the vasodilation induced by testosterone. Chou *et al.* (22) reported that the oestrogen receptor blocker ICI-182780 had no inhibitory effect upon testosterone-mediated dilatation of canine coronary arteries, and the studies of Yue *et al.* (21) and Tep-areenan *et al.* (26) both demonstrate that the vasodilatory efficacy of testosterone is not attenuated in the presence of the aromatase inhibitor aminoglutethimide. Furthermore, previous work by our group has demonstrated that the vasodilatory efficacy of oestradiol- 17β is significantly lower than testosterone in the pulmonary vasculature (37). Consequently, aromatase-mediated conversion of testosterone into oestradiol- 17β is unlikely to be involved in this vasodilatory response. The high concentration of testosterone required to induce vascular relaxation is mirrored by other non-genomic steroid signalling pathways (38–40), and such responses may well represent efficacy at a low affinity receptor – a number of which have recently been identified (41).

From these initial experiments looking at the vasodilatory capability of testosterone, an apparent difference in vascular reactivity to KCl and ACh in vessels harvested from Tfm and control vessels was observed. In the femoral arteries studied via wire myography, the initial contractions to KCl, which were utilised to ensure the viability of each vessel following dissection and mounting, were significantly reduced in the vessels obtained from Tfm mice. Similarly, the endothelial-dependent dilatation to ACh following precontraction with NA was also reduced in these vessels. Because of the reduction in response to ACh, we decided to repeat these experiments over the whole concentration–response range of ACh, and also NA, to determine whether there were any significant alterations in endothelial function and agonist-induced contraction. We also wanted to study the vessel compliance to determine whether there was any evidence of vascular remodelling.

Significant attenuation of the response to ACh (0.1 – 100 μ mol/l) was observed in the femoral arteries obtained from Tfm mice over all concentration ranges (Fig. 3). No significant alterations in the response to NA were observed (Fig. 4) although, as in the previous experiments, the initial exposures to KCl utilised to confirm vessel viability were significantly reduced. No significant alterations in femoral artery compliance were observed, although this was lower in the Tfm group. These findings contradict a recent study utilising the Tfm rat model that demonstrated a reduction in

vasoconstriction to NA and no change in vasoconstriction to KCl in thoracic aortae harvested from Tfm rats (42). However, this group also demonstrated that contraction to NA was dependent upon both sex and NO release (42), and the authors conclude that their results demonstrate that NO signalling is androgen receptor dependent, which is in agreement with the findings of the present study.

The observation that reduced vasodilatation to ACh occurred in the vessels harvested from Tfm mice demonstrates that endothelial function is impaired in these animals. In addition, the finding that vasoconstriction to KCl was also attenuated suggests that calcium channel function is also reduced in the Tfm mouse. NA and KCl both elicit smooth muscle contraction by triggering extracellular calcium entry following calcium channel activation, but vary in the sub-type of channel upon which they act. Addition of mmol/l concentrations of extracellular KCl disrupts the potassium concentration gradient across the smooth muscle cell membrane, causing the membrane to become depolarised, which activates voltage-operated calcium channels (VOCCs) (43). In contrast, NA elicits smooth muscle contraction via α -1 adrenoceptor stimulation, which results in G-protein coupled activation of receptor-operated calcium channels (ROCCs) (44). The fact that the response to NA was not affected but the response to KCl was significantly reduced in vessels harvested from Tfm mice would suggest that the function of ROCCs is unaffected but the function of VOCCs is impaired.

These data also suggest that testosterone-mediated vasodilatation does not occur via the release of endothelial-derived products, nor via inhibition of VOCCs. If vasodilatation to testosterone did occur via the release of endothelial-derived vasodilators, then it would be expected that the response to testosterone would be impaired, as with ACh. Similarly, if testosterone-mediated vasodilatation occurred via inhibition of VOCCs, then it would be expected that the response to testosterone would be enhanced, since VOCC channel function is already reduced. Because the response to testosterone was not altered in vessels from Tfm mice, this would suggest that these vasodilatory mechanisms are not involved in the acute response to testosterone, which is in agreement with previous studies (21, 26, 27). Clearly, further work is required to determine the underlying mechanism of testosterone-induced vasodilation.

However, whilst acute exposure to testosterone would appear not to modulate the release of endothelial-derived vasodilators or the function of VOCCs (as discussed above), clearly the chronic hypotestosteronaemia/androgen receptor deficiency exhibited in this model does. Such marked alterations in vascular reactivity may well manifest as cardiovascular disease in later life. Impairment of endothelial function is well recognised to be a characteristic of the diseased

coronary and systemic vessels common to CAD and peripheral vascular disease respectively, and a reduction in vasodilator capacity is proposed to be integral to the development of hypertension. Impaired calcium channel function is also likely to result in alterations in basal vascular tone, which may also be a contributory factor in the development of hypertension. Furthermore, elevated cholesterol levels may also result in the animals becoming prone to development of CAD. Recent studies from male patients with heart disease report a reduction in the circulating testosterone profile (reviewed in ref. 16), although it remains unknown whether this is a cause or effect of the disease state. The findings of the present study lend weight to the argument that the reduced levels of circulating testosterone may be a contributory factor to such conditions. Clearly, hypotestosteronaemia may indeed have serious consequences for the vasculature and may predispose to cardiovascular disease.

In summary, we have demonstrated that Tfm mice that lack a functional androgen receptor have significantly lower levels of circulating plasma testosterone and significantly elevated levels of serum cholesterol, compared with littermate control animals. These alterations in the circulating hormone profile are associated with a marked reduction in endothelial function, and impairment of the activity of VOCCs. This may have implications for the development of cardiovascular disease. The vasodilatory response to testosterone was unaffected, demonstrating no involvement of the androgen receptor, and implying the lack of involvement of endothelial-derived vasodilators or inhibition of VOCCs in this response. Because the Tfm mouse has a reduced level of circulating testosterone coupled with the lack of a functional androgen receptor, further experiments are required to determine which of these two characteristics are responsible for the observed alterations in endothelial and vascular function, and of the precise cellular mechanisms involved in producing these potentially deleterious vascular effects.

Acknowledgements

The authors wish to thank Mr Martin Loxley, Department of Clinical Chemistry, Royal Hallamshire Hospital, who organised the analysis of total cholesterol, progesterone and cortisol levels.

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Received 4 July 2002

Accepted 25 September 2002