

Testosterone suppresses oxidative stress via androgen receptor-independent pathway in murine cardiomyocytes

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Abstract. Evidence supports that oxidative stress exerts significant effects on the pathogenesis of heart dysfunction. On the other hand, the presence of specific androgen receptor (AR) in mammalian cardiomyocytes implies that androgen plays a physiological role in cardiac function, myocardial injury and the regulation of the redox state in the heart. This study used the testicular feminized (Tfm) and castrated male mice to investigate the effects of testosterone deficiency, physiological testosterone therapy and AR on oxidative stress in cardiomyocytes. Tfm mice have a non-functional AR and reduced circulating testosterone levels. Male littermates and Tfm mice were separated into 5 experimental groups: non-castrated littermate controls, castrated littermates, sham-operated Tfm, testosterone-treated castrated littermates and testosterone-treated sham-operated Tfm mice. Cardiomyocytes that were isolated from the left ventricle were used for determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) enzyme activities, and malondialdehyde (MDA) levels. Additionally, mitochondrial DNA (mtDNA) deletion mutations were detected by nested PCR. The SOD and GSH-Px enzyme activities of cardiomyocytes were decreased, and the MDA levels and the proportion of mtDNA mutations were increased in castrated and sham-operated Tfm mice compared to control mice. However, an increase was observed in the activities of SOD and GSH-Px enzyme as well as a decrease in MDA levels and the proportion of mtDNA mutations in the mice that had received testosterone therapy. These changes were statistically similar in castrated and sham-operated Tfm

mice after testosterone therapy. In conclusion, it is testosterone deficiency that induces oxidative stress in cardiomyocytes. Physiological testosterone therapy is able to suppress oxidative stress mediated via the AR-independent pathway.

Introduction

Testosterone is the major male androgen and deficiencies in circulating levels are a prominent feature observed in aging males. Testosterone exerts a variety of anabolic and androgenic effects on many organs, most of which are mediated by the nuclear androgen receptor (AR). The AR is expressed in mammalian and primate cardiomyocytes, suggesting that androgens may play a role in normal heart function (1,2). Low testosterone levels or androgen deficiency have been found to increase cardiovascular risk factors and produce adverse effects on cardiac function (2,3). Furthermore, testosterone therapy in humans (4) and rats (5) with low testosterone levels has been shown to improve cardiac function.

Substantial evidence supports the view that oxidative stress may exert effects on the pathogenesis of heart dysfunction (6). Cardiomyocytes are rich in mitochondria that can provide rapid and substantial ATP production to meet increased energy demands for increased work. However, this increased energy production can lead to an increased reactive oxygen species (ROS) generation that can surmount antioxidant defenses and lead to the intrinsic generation of oxidative stress (7). Low concentrations of ROS are necessary to the normal function of cellular activities and can regulate enzyme activity, signal transcription, and gene expression. However, at high concentrations they may damage cellular components, including DNA, RNA, proteins and lipids (8). Not only are mitochondria the major source of ROS, but they are also immediate targets of the pathological insult. Oxidative damage to mitochondrial DNA (mtDNA) by ROS may induce nucleotide and mtDNA mutations. Accumulation of mutations and oxidative damage to mtDNA may result in increased ROS in mitochondria and induction of further mtDNA mutations (9,10).

Bekesi *et al* reported that testosterone treatment decreases superoxide anion production in human neutrophils, suggesting

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a possible antioxidant effect (11). In contrast, testosterone may directly induce oxidative stress in mammalian tissues (12). Little is known of the effects of testosterone on the regulation of oxidative stress in the heart. This experimental design allowed us to assess the effect of testosterone deficiency and physiological testosterone therapy on the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzyme, levels of oxidative damage in lipids quantified by malondialdehydes (MDAs), and the proportion of mtDNA deletion mutations in murine cardiomyocytes. Other studies have demonstrated that testosterone modulates the production of ROS in cerebellar granule cells via an AR mediated mechanism (13). Hence, we assessed whether the effects of testosterone on the above-mentioned changes were mediated via AR-dependent pathways.

Materials and methods

Experimental animals and design. All experimental procedures were approved by the Animal Research Committee of Nanfang Hospital, Southern Medical University. Breeder mice were purchased from the Model Animal Research Center of Nanjing University (female, $A^{W-J}/A^{W-J} Eda^{Ta-6J} +/+Ar^{Tfm}$; male, $A^{W-J}/A^{W-J} Eda^{Ta-6J} +/Y$). Male littermate and the testicular feminized (Tfm) mice were bred in our laboratory. The gender of the Tfm mice was determined by PCR. Tfm mice exhibit an X-linked, single-base-pair deletion in the gene encoding the AR. This deletion results in premature termination of AR protein synthesis, which in turn leads to a non-functional AR (14,15). In addition, circulating levels of testosterone are reduced in the Tfm mouse, reportedly 10-fold lower compared to its male littermate (15).

At 8 weeks of age, male littermate and Tfm mice underwent either surgical castration or sham operation, respectively. Four weeks following operation, castrated mice were treated with (n=8) or without (n=8) testosterone propionate (Guangzhou Mingxing Pharmaceutical Co.; 070701), while sham-operated Tfm mice were also treated with (n=8) or without (n=7) testosterone propionate. Testosterone was injected intramuscularly at 3 mg/kg body weight, diluted in sesame oil, once every 72 h for 3 months. Age-matched 24-week-old non-surgical littermates were used as the control (n=8). Pharmacokinetic determination of physiological testosterone was conducted before these manipulations.

Pharmacokinetic determination of physiological testosterone therapy. To establish and maintain a dosing regimen of testosterone to physiological concentrations, we first had to determine the appropriate volume and frequency of 25 mg/ml testosterone propionate. At 8 weeks of age, male littermate mice (n=36) underwent surgical castration and were then allowed to recover in individual cages for 4 weeks. Consequently, 12-week-old mice received a single 3 mg/kg intramuscular injection of testosterone propionate (the human replacement dose of testosterone propionate is 0.35 mg/kg administered 2-3 times per week). Mice were sacrificed in serial intervals at 30 min and at 1, 2, 4, 8, 16, 24, 32, 48 and 72 h after injection, and testosterone concentrations were measured. To ensure reproducibility, 18 additional castrated littermates received a second-cycle injection (double administration) beginning at

72 h after the first and were sacrificed at 1, 2, 4, 8, 24 and 72 h after the second injection. At the same time, in order to determine normal testosterone concentrations, 9 non-castrated male mice were sacrificed at 00:00, 08:00 and 16:00 h.

Isolation of cardiomyocytes. Left ventricular myocytes were enzymatically isolated from 39 male mice as previously described (16). The solutions were supplements of modified commercial minimal essential medium (MEM) Eagle-Joklik. The washing solution contained HEPES-MEM with the addition of 0.5 mM EGTA. The resuspension medium contained HEPES-MEM supplemented with 0.5% BSA and 0.3 mM $CaCl_2$. Briefly, the hearts were quickly removed and connected to a plastic cannula for retrograde perfusion through the aorta. The cell isolation procedure consisted of 3 main steps: i) for low-calcium perfusion, blood washout in the presence of EGTA was performed for about 10 min, and collagenase (selected type I, Worthington Biochemical Corp.) perfusion of the myocardium was carried out at 37°C with HEPES-MEM gassed with 85% O_2 , 15% N_2 , ii) the heart was removed from the cannula, the left ventricle was cut into small pieces and subsequently shaken in resuspension medium at 37°C. Supernatant cell suspensions were washed and resuspended in resuspension medium, and iii) intact cells were enriched by centrifugation. This procedure was repeated 4-5 times.

Preparation of homogenates. Freshly isolated cardiomyocytes (5×10^6) were mixed with 1 ml of cell lysis solution, repeatedly pipetted and sonicated in ice. In order to eliminate debris, the crude homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then used for the assay of SOD, GSH-Px and MDA.

Assay of antioxidant enzyme activity. The activities of SOD and GSH-Px enzyme were determined using assay kits (Jian Cheng Biology Research Center, China). SOD activity was measured through the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition in 1 ml reaction solution. GSH-Px activity was measured by using H_2O_2 and GSH as substrates. One unit of GSH-Px activity was defined as the amount of enzyme required to degrade 1 mM of GSH per min subtracting non-enzymatic reaction. Each end-point assay was monitored by absorbance at 550 nm in SOD activity and at 412 nm in GSH-Px activity. Activity was expressed as U/ml.

MDA assay. MDA levels were determined by the thiobarbituric acid reactive substances (TBARS) assay using a MDA Assay kit (Jian Cheng Biology Research Center). TBARS is widely used to quantify the lipid peroxidation caused by high ROS levels. The concentration of TBARS was measured as a product of lipid peroxidation (17). Each end-point assay was monitored using absorbance at 532 nm and the results were expressed as nmol/ml.

mtDNA mutation assay. As previously described (18,19), a nested PCR method was used to determine mtDNA deletion mutations in experimental DNA samples extracted from isolated cardiomyocytes.

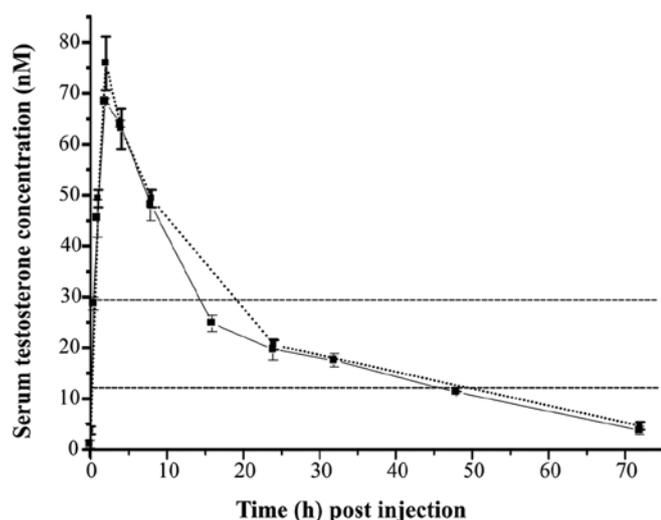


Figure 1. Serum concentration of total testosterone in castrated littermates after a single (solid line) and double (dashed line) 72 h intramuscular injection of testosterone propionate. The area between the dashed parallel lines denotes the physiological range of testosterone (12.6-28.7 nM).

The primer sequences were as follows: F1, taattcaagcctagc tattc (8549-8569); F2, gggatgttttaggcttagg (13364-13383); F3, caagtcacatgaccattaactgg (8644-8665); F4, gattttatgggtgtaatg (13338-13357); and F5, catgatctaggaggctgctgacctc (8934-8958) (19). Nested PCR conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation, annealing and extension at 94°C for 30 sec, 49°C for 30 sec and 72°C for 90 sec, and a final extension at 72°C for 10 min. Primers F1 and F2 were used as outer primers for the first 30 cycles, then 2 μ l of products of the first reaction were used with inner primers F3 and F4 for the second 30 cycles. Primers F2 and F5 were used to determine the relative quantification of wild-type mtDNA without deletions in each sample. The PCR condition consisted of 5 min at 95°C, followed by 30 cycles of 45 sec at 95°C, 45 sec at 49°C and 5 min at 72°C, and a final extension at 72°C for 10 min. Amplified products were electrophoresed through 1.5% agarose gels and stained with ethidium bromide. The wild-type and deleted mtDNA could be detected with this method in one system. The gel was visualized on a transilluminator and photographed with a camera. M represented the sum of integrated optical density (IOD) values of deleted bands. W represented the IOD values of undeleted bands serving as the internal control. The proportion of mtDNA deletion mutations = $M/(M+W)$ (20). Products were purified and sequenced (Invitrogen Biotechnology Co.).

Statistical analysis. SPSS Version 13.0 (Statistical Software for Social Sciences, Chicago, IL) was used for statistical analysis. Results were expressed as the means \pm SD. Data were subsequently analyzed by one-way ANOVA, assumed with LSD and Dunnett's T3 multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Determination of a dosing regimen to physiological testosterone levels in castrated mice. The normal range

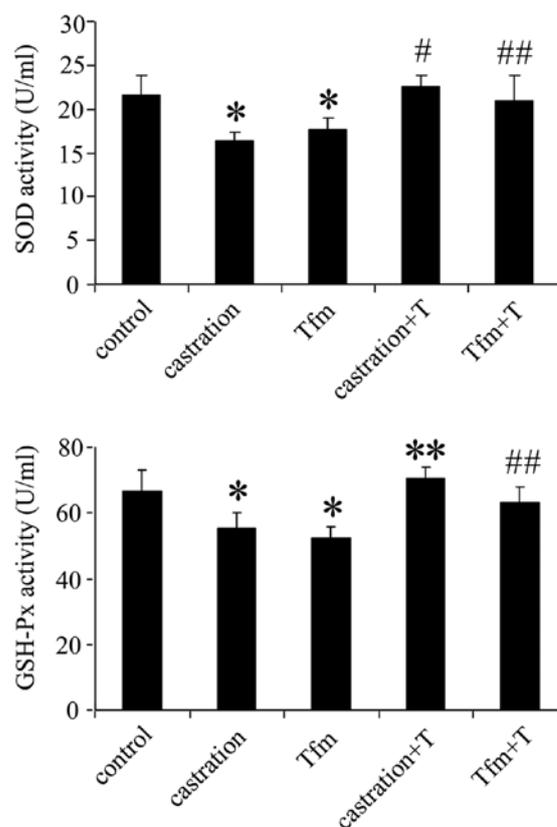


Figure 2. SOD and GSH-Px activities in cardiomyocytes. The values represent the means \pm SD. * $P < 0.05$ vs. control; # $P < 0.001$ vs. castrated group; ** $P < 0.01$ vs. castrated group; ## $P < 0.05$ vs. Tfm group. T, testosterone.

of testosterone concentrations was from 12.6 to 28.7 nM. Baseline levels of serum testosterone after castration were about 6% of normal levels. The levels rose significantly from baseline after the 3 mg/kg intramuscular testosterone injection at 30 min, reached a peak at 2 h, and remained at the normal range between 14 and 50 h (Fig. 1). The mean area under the curve (AUC) for the 72 h period was 20.6 nM. A similar window of activity was observed after the second 72 h injection. The mean AUC for the second 72 h period was 22.7 nM. Pharmacokinetics showed that there was no statistical difference for testosterone concentrations between single and double administrations. Furthermore, after both administrations, the mean AUC was within the normal range. These results are in agreement with the study of Nettleship *et al* (21) and indicate a good reproducibility of the pharmacokinetic profile and a sufficient physiological replacement therapy of 3 mg/kg/72 h intramuscular injection of testosterone propionate in castrated male mice.

Effect of testosterone on antioxidant enzyme activity in murine cardiomyocytes. As shown in Fig. 2, a significant decrease of the SOD and GSH-Px specific activities was observed in cardiomyocytes isolated from castrated and sham-operated Tfm mice compared to the age-matched control group ($P < 0.05$). No significant differences were observed between the two experimental groups (castrated mice vs. sham-operated Tfm mice, $P > 0.05$). Testosterone treatment increased

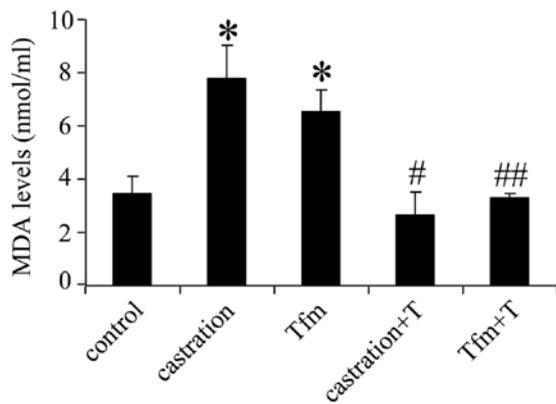


Figure 3. MDA levels in cardiomyocytes. The values represent the means \pm SD. * P <0.01 vs. control; # P <0.001 vs. castrated group; ## P <0.01 vs. Tfm group. T, testosterone.

SOD and GSH-Px activities in castrated and sham-operated Tfm mice compared to untreated mice (P <0.05). Furthermore, these levels were similar in testosterone-treated castrated and sham-operated Tfm mice (P >0.05).

Effect of testosterone on MDA levels in murine cardiomyocytes. Additionally, a significant increase in the MDA levels was found in cardiomyocytes isolated from castrated mice and sham-operated Tfm mice compared to the age-matched

control group (P <0.01). No significant differences were observed between the two experimental groups (castrated mice vs. sham-operated Tfm mice, P >0.05). Testosterone treatment resulted in a similar decrease in MDA levels in both castrated and sham-operated Tfm mice (Fig. 3), compared to untreated mice (P <0.01).

Effect of testosterone on the proportion of mtDNA deletion mutations in murine cardiomyocytes. PCR products of length 989-, 846-, 476- and 303-bp were directly sequenced. The product size, deletion size, junction sites and repeat length of these four observed deletions are presented in Table I. These common amplification products occurred between directly repeated sequences in the mtDNA.

A product of 4549 bp in length was detected in all groups using standard PCR conditions, representing amplification of wild-type mtDNA without deletions. An increased proportion of mtDNA deletion mutations was observed in castrated and sham-operated Tfm mice and these values were statistically similar (0.49 ± 0.02 vs. 0.52 ± 0.04 ; P >0.05). The levels were significantly higher than those observed in the control mice (0.26 ± 0.05 ; both P <0.001). After testosterone treatment, a decrease in mtDNA deletions was found in both castrated and sham-operated Tfm mice compared to the untreated mice (0.38 ± 0.02 vs. 0.49 ± 0.02 and 0.35 ± 0.06 vs. 0.52 ± 0.04 , respectively; both P <0.01). Moreover, these values were similar in testosterone-treated castrated and sham-operated Tfm mice (0.38 ± 0.02 vs. 0.35 ± 0.06 ; P >0.05) (Fig. 4).

Table I. Structure of mouse mtDNA deletions.

Deletion size (bp)	Breakpoints	Direct repeat (bp)	Repeated sequences	Product size (bp)
3726	9553/13279	14	AAGCAAATCCATAT	989
3867	9089/12956	15	AGCCCTACTAATTAC	846
4236	8884/13120	13	TCTTTGCAGGATT	476
4414	8758/13172	11	TCCTACAATA	303

mtDNA, mitochondrial DNA.

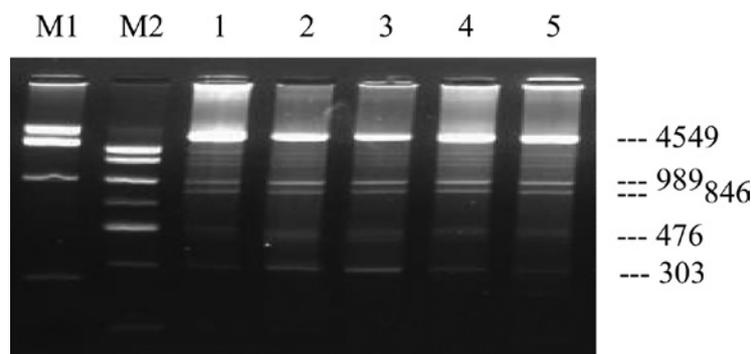


Figure 4. Detection of multiple mtDNA deletions in cardiomyocytes. Line 1, control group; Line 2, castrated group; Line 3, Tfm group; Line 4, castrated + testosterone group; Line 5, Tfm + testosterone group. Numbers on the right side of the image indicate the size of the expected product. M1 marker, fragments of size 5000, 2500, 1000 and 250 bp are visible in order from the top of the lane; M2 marker, fragments of size 2000, 1500, 1000, 700, 500, 300 and 100 bp.

Discussion

We found that testosterone concentrations in castrated mice were 94% lower than in control mice. Testosterone concentrations in sham-operated Tfm mice were 89% (data not shown), a result also reported by Jones *et al* (15). Testosterone therapy restored physiological testosterone levels in castrated and Tfm mice, and finally, oxidative damage that occurred in cardiomyocytes after testosterone deficiency and testosterone therapy was observed.

Antioxidant defenses, both enzymatic and non-enzymatic, protect the cell from ROS that can cause oxidative damage (22). The cellular antioxidant defense system consists of SOD, GSH-Px and other related enzymes (23). This dynamic system must be precisely regulated in order to protect cells from oxidative damage. The present study revealed that left ventricular myocytes from castrated and sham-operated Tfm mice had a decrease in SOD and GSH-Px enzyme activities compared to the control mice. Moreover, the parameter was statistically similar in these two groups. These observations provide evidence that testosterone deficiency contributes to the deterioration of antioxidant defense mechanisms in cardiomyocytes. In this respect, the results of our study are consistent with those reported by Barp *et al* (24) and Kłapcińska *et al* (25) in hearts and Meydan *et al* (23) in the hippocampal tissue. Data regarding the potential antioxidant role of exogenous testosterone in the heart are very scarce, and conflicting results have been observed in different tissues. Kłapcińska *et al* (25) reported that testosterone replacement in castrated rats exacerbated the decline in cardiac muscle antioxidant defense, including SOD and GSH-Px activities. In the hippocampus, exogenous testosterone increased SOD and GSH-Px enzyme activities which were reduced after castration (23). Marin *et al* (3) found that the activities of total SOD and GSH-Px were unaffected in neutrophils after testosterone treatment. In the present investigation, long-term physiological testosterone therapy significantly increased SOD and GSH-Px activities, suggesting an improvement of antioxidant activity. The discrepancies between our results and the results of others may be attributed to the fact that those experiments were performed using tissue lysates, in which case other cell types are present and could mask changes occurring in ventricular cardiomyocytes. The importance of this is paramount as non-cardiac cells account for about twice the number of ventricular cells compared to cardiomyocytes. Furthermore, after comparing our results to those found in other tissues, we suggest that the effect of testosterone is tissue-dependent (12).

Membrane lipids, which are rich in polyunsaturated fatty acids, are vulnerable to peroxidation by oxidants. The level of MDA, which occurs due to lipid peroxidation, is an indicator of oxidative damage. Lipid peroxidation has been shown to increase in cardiac mitochondria during oxidative damage (26,27). We observed that cardiomyocyte MDA levels significantly increased in castrated and sham-operated Tfm mice compared to control mice. This increase demonstrates that oxidative damage occurs in cardiomyocytes as a result of lipid peroxidation attributed to testosterone deficiency. In agreement with our findings, castration leads to higher MDA content (25) and an increase in lipid peroxidation (28) in the left ventricle. Androgens have been shown to effectively

prevent the formation of lipid peroxidation (29). It has been reported that testosterone administration leads to a decrease in MDA levels in rat liver (30) and brain (23,31). In the study by Sreelatha Kumari *et al*, administration of testosterone in castrated rats partially reversed the increase in lipid peroxidation in the hearts (28). However, Kłapcińska *et al* (25) found that testosterone replacement in castrated male rats led to a further increase in MDA levels in the left ventricle. The reason for this discrepancy is unclear. Our study indicates that testosterone therapy causes a decrease in the MDA levels in cardiomyocytes, which are increased by testosterone deficiency.

mtDNA is especially susceptible to oxidative damage due to its proximity to the inner mitochondrial membrane where oxidants are generated, and to the lack of protective histones and DNA repair activity (32). DNA damage in postmitotic tissues, including the heart, is more extensive than that observed in mitotic tissues, such as the liver, since cardiomyocytes have limited ability to undergo mitosis (33). Oxidative damage has been proposed to involve single- or double-stranded DNA breaks, leading to mtDNA deletions. mtDNA deletions are flanked by exact and inexact direct repeats. Three mutant mtDNAs with 3726-, 3867- and 4236-bp deletions are most often observed in the aging murine heart, brain, kidney, liver and cardiomyocytes (18-20,34). In the study by Wang *et al*, the 4056 bp deletion was often found in aged tissues and was associated with directly repeated sequences in the mtDNA (19). Similarly, the deletion sizes of 3726-, 3867- and 4236-bp in length were amplified in both controlled and experimental cardiomyocytes in our study. Another deletion observed in mtDNA was 4414 bp, which was associated with 11 bp direct repeats, supporting the view that large-scale deletions most frequently occur at direct repeat sequences (18,19,34). Although we were unable to determine the concentration of the mtDNA genome, the wide-type internal control allowed for a comparison of the relative quantitation of deleted mtDNA molecules. Testosterone deficiency, described by castration and Tfm, resulted in higher proportions of mtDNA deletions compared to normal testosterone levels, while testosterone administration partially reversed these increases. In our experiment, we estimated that multiple deletion products were amplified in all experimental groups due to a higher DNA input (500 ng) (19) or the effect of isolation procedure. There are few studies on mtDNA deletion mutations induced by testosterone. It is possible that testosterone stimulates the alternation of ROS production, thereby dramatically changing the amount of mutations in mtDNA.

As previously mentioned, we observed a protective role of testosterone therapy against oxidative stress. The amelioration was statistically similar in castrated and sham-operated Tfm mice after testosterone therapy. Due to the following observations we conclude that the positive effects of testosterone on oxidative stress occur via an AR-independent pathway: i) Tfm mice carry non-functional AR and still respond to testosterone, and ii) castrated mice with functional AR show signs of oxidative stress that can be cured with testosterone. Research concerning the effects of testosterone on the production of ROS in the heart does not explain the mechanism behind the observed results (25,28). Ahlbom *et al* (13) and Lin *et al* (35) found that oxidative stress modulated by testos-

terone in cerebellar granule cells and prostate cancer cells was mediated via an AR mediated mechanism. It is difficult to say why we could not replicate the previously obtained results. As mentioned above, testosterone has a tissue-dependent effect, and we presume that its antioxidant properties may be tissue-dependent and involve different signaling pathways in cardiomyocytes. We hypothesize that aromatization to 17 β -estradiol could occur and that the effects of testosterone treatment could then be mediated through different receptors.

In conclusion, our results support the idea that testosterone deficiency contributes to oxidant stress in Tfm and castrated male mice cardiomyocytes. This study has demonstrated a beneficial effect of physiological testosterone on oxidant stress, an action that is independent of the typical AR. Reduced concentrations of testosterone in the elderly and in some pathological conditions may contribute to the development of heart disease through the induction of oxidative stress. The maintenance of physiological testosterone concentrations may maintain the redox balance in murine cardiomyocytes.

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