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Testosterone protects cardiac myocytes from superoxide injury via NF-κB signalling pathways

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ABSTRACT

Aims: Cellular and molecular mechanisms underlying the effects of androgenic hormone testosterone on the heart remain unclear. This study examined the impact of testosterone on viability of cardiac myocytes and the role of NF-κB signalling pathways.
Materials and methods: Rat H9c2 myocytes were cultured in steroid-free media and incubated with hydrogen peroxide (H_2O_2, 200 μM, 6 h). NF-κB expression was knocked down by RelA (p65) siRNA interference. Testosterone (5–100 nM, 24–48 h) was provided into the media and androgen receptor (AR) blocked by flutamide (100 nM). Cell apoptotic/necrotic death was determined by morphological examination and flow-cytometric analysis. Gene expression was examined by Western blotting analysis.
Key findings: Testosterone supplements reduced the superoxide-induced apoptotic/necrotic death, stimulated NF-κB (RelA) expression, activated Akt activity, and inhibited Caspase-3 expression in the cardiac myocytes. The hormonal effects were abolished by either AR blocker flutamide or NF-κB-knockdown. Testosterone also induced ERK1/2 activation, which was not affected by flutamide or NF-κB knockdown, and blocking the ERK activity did not affect the protective effect of the hormone on the cells.
Significance: This study demonstrates that exogenous testosterone supplementation protects cardiac myocytes from superoxide injury via AR mediation and dependent on normally functional canonical NF-κB (RelA/p50) signalling pathways. The NF-κB signalling may be an important key molecular basis for myocardial benefits of hormone (testosterone) therapy.

1. Introduction

Deficiency of androgenic hormones is associated with reduced quality of life and longevity and a higher risk of cardiovascular disease (CVD) [12,14]. Androgen replacement therapy, which restores the physiological hormonal levels through supplementation of exogenous testosterone, can improve clinical symptoms associated with androgen deficiency. The therapy has been available to hypogonadal men for over three decades and more recently has occasionally also been given to postmenopausal women following oophorectomy. Although epidemiological data suggest that testosterone therapy may reduce components of the metabolic syndrome, the extent to which it provides direct cardiac protection remains uncertain [3] and to date, the clinical application for the prevention of CVD remains controversial.

Heart failure is a major contributor to death from CVD. The progression from cardiac injury to symptomatic heart failure is largely attributable to loss of functional cardiomyocytes through pathways of cell death, resulting in replacement of myocytes by scar tissue. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a family of phylogenetically conserved proteins that act as nuclear transcription factors, is known to play key roles in the regulation of cardiac survival, apoptosis, hypertrophy and pathological remodelling during ischaemic injury [10]. There are two – canonical (RelA/p50 dimer) and noncanonical (RelB/p52 dimer) – signalling pathways of NF-κB, with RelA/p50 the predominant complex in the heart. Studies have documented the role of NF-κB signalling in the regulation of cardiac survival through repression of apoptotic cell death undergoing disease conditions [2,16,18,19,22]. However, chronic or long-term activation of NF-κB may induce expression of inflammatory cytokines and produce cardiac cell death [4,9].

Testosterone is the most important androgen for the physiology in man. Most (over 90%) testosterone directly binds and activates intracellular androgen receptor (AR). It can also act directly on specific cell membrane binding sites (SMBS), a non-AR-mediated pathway [12]. Cardiac myocytes contain functional AR and SMBS and are therefore targets for hormonal action. Epidemiological studies demonstrate an increased risk of heart failure due to deficiency of physiological androgens, as occurred in ageing men [14], prostate cancer patients with androgen deprivation therapy [15], as well as in women with bilateral

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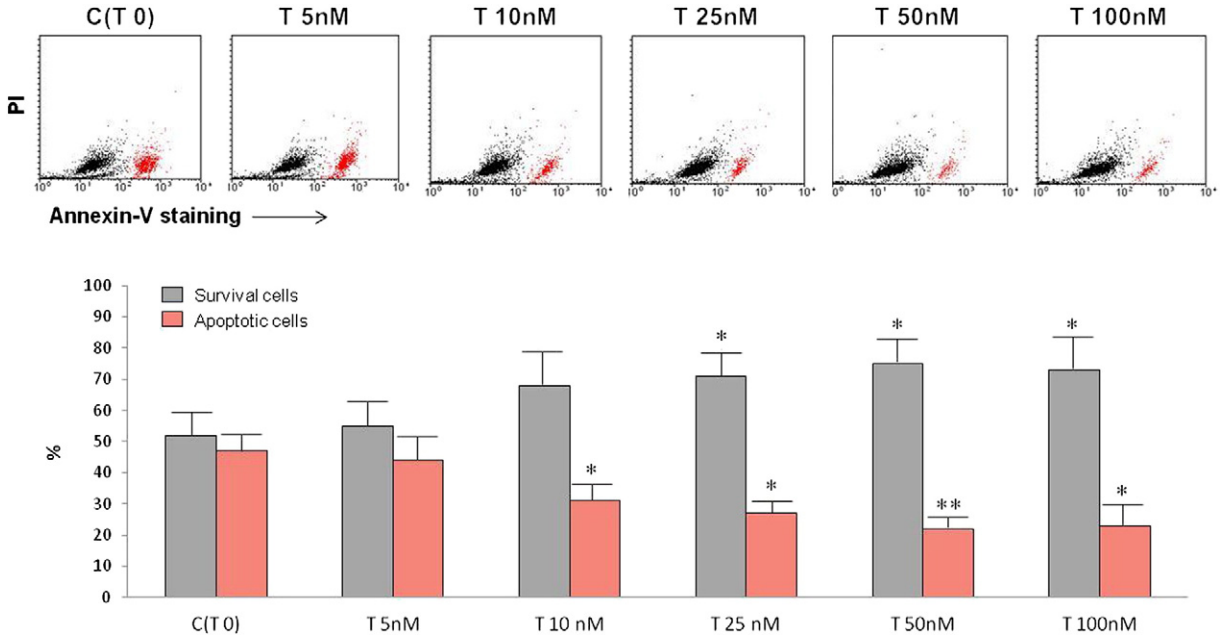

Fig. 1. Testosterone improves viability of cardiac myocytes undergoing superoxide injury. H9c2 cells were incubated with testosterone (T, 5–100 nM) for 24 h, suffered to hydrogen peroxide (H_2O_2) treatment (200 μM) for 6 h, then stained with Annexin-V/PI and assessed by FACS. Dotplots: from a representative experiment; apoptotic cells characterised by Annexin-V positive and PI negative staining (indicated in red colour). Bar graph: Mean ± SD of the percentage of survival and apoptotic cells from three independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. C (T 0).

oophorectomy [12]. In castrated rats [1,20], the androgen deficiency worsened myocardial injury and reduced ejection fraction and diastolic dysfunction, and the cardiac function was improved with testosterone replacement. Testosterone can enhance cardiac hypertrophy and remodelling of the heart, resulting in either improvement of cardiac function [23] or increasing the risk of acute cardiac rupture then worsening of cardiac function [5]. The effects of testosterone on cardiac cells are many and varied, with different molecular signalling pathways involved and to date, remain unclear. In this study, we have examined the impact of testosterone supplementation on the viability of cardiac myocytes, with particularly focusing on the NF-κB signalling pathway.

2. Materials and methods

2.1. Cell cultures

Myocardiac H9c2 cell (Catalog. No. CRL-1446), a clonal line derived from embryonic rat heart, was from American Type Culture Collection (ATCC, Manassas, VA). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Co., St. Louis, MI) with D-glucose at 4.5 g/L, 10% foetal bovine serum (FBS), 10,000 U/L penicillin, and 10 mg/L streptomycin, in an incubator with 5% CO_2, at 37 °C and the medium was changed every 2 days. When confluence reached the cells (between passages 3 and 5) they were subcultured by detaching with 0.25% trypsin–EDTA solution (Sigma Co.) and re-seeding into new plates at a ratio of 1:5 in DMEM with 10% steroid-free FBS (Sigma Co.). Cells at ~75% confluence were cultured in serum-free DMEM, treated with the hormone and its receptor blocker (see below), and subjected to hydrogen peroxide (H_2O_2, 200 μM, 6 h) treatment.

2.2. Testosterone and androgen receptor (AR) blocker

Testosterone (cat# T1500) and AR blocker flutamide (cat# F9397) were purchased from Sigma Co. and dissolved in 100% ethanol for storage. For the study they were diluted with PBS freshly before application in each experiment and added into the cell culture media with the final concentration of ethanol at 0.1%. Testosterone was added into the media at concentrations of 5 to 100 nM for 24 to 48 h and flutamide (100 nM) added into the media at 3 h before the hormone.

2.3. Knockdown of NF-κB gene expression

Expression of nuclear NF-κB RelA (p65) in H9c2 myocytes was knocked down by RNA interference using a RelA/p65 siRNA Transfection Kit (GeneResearch Co. Australia) that contained SignalSilence® NF-κB p65 siRNA I (2 μM, mouse specific) and Thermo Scientific DharmaFECT® 1 siRNA transfection reagent. As testing in cultured H9c2 cells by the manufacturer, siRNA (100 nM) complexed with the reagent resulted in silencing (reduction) of mRNA (in QuantiGene® branched DNA/RNA assay) at >90% with little toxicity (cell viability >95% in the alamarBlue® assay). In this study siRNA transfection was performed in accordance with the manufacturer's protocol optimised for use with H9c2 cells in culture. In brief, cells were washed with serum-free DMEM, incubated with the transfection reagent (7 μL in 343 μL DMEM) containing RelA/p65 siRNA (100 nM) for 2–4 h, and continued to culture in serum (10%, steroid-free)-enriched DMEM with the siRNA for 48 h before being ready for the hormonal testing.

2.4. Giemsa staining and morphological analysis

H9c2 cells cultured on cover glasses were washed with ice-cold PBS, fixed with methanol for 5 min, stained with Giemsa staining solution that was freshly prepared by 1:20 dilution of Giemsa stock solution (Sigma Co.) for 15–20 min, checked by microscopy until an ideal stain was obtained, and mounted with glycerol gel. The cells were then examined under a light microscope (×200 and ×400); both normal survival and apoptotic/dead cells, characterised by nuclear fragmentation and condensation (at a relatively early stage) or by nuclear disruption (at a late stage) were counted in five different fields randomly in each experiment and the percentage was calculated in each group.