Physiological testosterone attenuates profibrotic activities of rat cardiac fibroblasts through modulation of nitric oxide and calcium homeostasis

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Abstract. Testosterone deficiency is associated with poor prognosis among patients with chronic heart failure (HF). Physiological testosterone improves the exercise capacity of patients with HF. In this study, we evaluated whether treatment with physiological testosterone contributes to anti-fibrogenesis by modifying calcium homeostasis in cardiac fibroblasts and we studied the underlying mechanisms. Nitric oxide (NO) analyses, calcium (Ca2+) fluorescence, and Western blotting were performed in primary isolated rat cardiac fibroblasts with or without (control cells) testosterone (10, 100, 1,000 nmol/L) treatment for 48 hours. Physiological testosterone (10 nmol/L) increased NO production and phosphorylation at the inhibitory site of the inositol trisphosphate (IP3) receptor, thereby reducing Ca2+ entry, phosphorylated Ca2+/calmodulin-dependent protein kinase II (CaMKII) expression, type I and type III pro-collagen production. Non-physiological testosterone-treated fibroblasts exhibited similar NO and collagen production capabilities as compared to control (testosterone deficient) fibroblasts. These effects were blocked by co-treatment with NO inhibitor (L-NG-nitro arginine methyl ester [L-NAME], 100 μmol/L). In the presence of the IP3 receptor inhibitor (2-aminoethyl diphenylborinate [2-APB], 50 μmol/L), testosterone-deficient and physiological testosterone-treated fibroblasts exhibited similar phosphorylated CaMKII expression. When treated with 2-APB or CaMKII inhibitor (KN93, 10 μmol/L), testosterone-deficient and physiological testosterone-treated fibroblasts exhibited similar type I, and type III collagen production. In conclusion, physiological testosterone activates NO production, and attenuates the IP3 receptor/Ca2+/entry/CaMKII signaling pathway, thereby inhibiting the collagen production capability of cardiac fibroblasts.

Key words: Fibroblasts, Testosterone, Ca2+/calmodulin-dependent protein kinase II, Nitric oxide, Inositol trisphosphate

TESTOSTERONE DEFICIENCY is a common manifestation in men with heart failure (HF) [1]. Testosterone deficiency increases mortality and hospitalization risks and reduces the exercise capacity in patients with chronic HF [2-5]. In men with HF, testosterone deficiency is associated with diastolic dysfunction [6], which is regulated by collagen accumulation in the myocardium [7]. Multiple clinical HF trials have proven that physiological testosterone replacement improves exercise capacity [8-12]. Cardiac fibrosis is associated with poor HF prognosis and low exercise capacity [13, 14]. Physiological testosterone can reduce the collagen production capability of cardiac fibroblasts and attenuate cardiac fibrosis in HF, aging, and castrated animals [15-18]. However, the mechanisms underlying the antifibrotic effects of physiological testosterone warrant further elucidation.

Physiological testosterone can increase nitric oxide (NO) levels in aortic endothelial cells [19] and reduce...
calcium (Ca²⁺) mobilization, involved in pro-fibrotic signaling [20], in smooth muscle cells [21]. The purpose of the current study was to clarify whether physiological testosterone regulates cardiac fibrogenesis by inhibiting the Ca²⁺ signaling pathway through the activation of NO signaling and to study the underlying mechanisms.

**Materials and Methods**

**Cardiac fibroblast isolation**

The study was approved by the local ethics review board (approval number: LAC-2019-0508). All animals received human care. Cardiac fibroblasts were isolated from male Sprague-Dawley rats (weighing 300–350 g) and cultured in Dulbecco’s modified Eagle’s medium by using a method described previously [18]. In brief, the hearts were mounted on a Langendorff apparatus and perfused with phosphate-buffered saline (PBS) containing 0.02% collagenase (Sigma, St. Louis, MO, USA) at 37°C for 35 minutes. Then, the left ventricle was chopped and shaken in PBS until single fibroblasts were obtained. The vimentin-positive and CD31-negative cells were sub-cultured and plated at a density of 1 × 10⁶ cells/cm² on culture dishes. Cardiac fibroblasts were then cultured with serum-free medium for 24 hours before each treatment.

**NO measurement**

For NO measurement, DAF-2 DA was performed through fluorescence microscopy as previously described [22]. In brief, testosterone-deficient cardiac fibroblasts (control cells) were treated with or without testosterone (10, 100, 1,000 nmol/L) for 24 hours. The fibroblasts were stained with DAF-2 DA (5 μmol/L, Abcam, Cambridge, UK) for 10 minutes in the dark and then washed with PBS. Fluorescence imaging was performed using an Evos FL microscope (Thermo Fisher Scientific, Pittsburgh, PA, USA) with an excitation wavelength of 470 nm and emission wavelength of 515 nm. Protein content from the cell lysate of each treatment was used for normalization.

**Western blotting**

Western blotting was performed as described previously [23]. In brief testosterone-deficient cardiac fibroblasts (control cells) were incubated in serum-free medium for 24 hours followed by treatment with or without testosterone (10, 100, 1,000 nmol/L, Sigma), NO inhibitor (L-NG-nitro arginine methyl ester [L-NAME], 100 μmol/L, Sigma), inositol trisphosphate (IP3) receptor inhibitor (2-aminoethyl diphenylborinate [2-APB], 50 μmol/L, Sigma), or Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) inhibitor (KN93, 10 μmol/L, Sigma) for 48 hours. Cells of interest were lysed in radioimmunoprecipitation assay buffer in the presence of 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, 50 mmol/L Tris pH 7.4, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor cocktails (Sigma). The proteins were fractionated using 10% SDS-polyacrylamide gel electrophoresis and transferred onto an equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were then incubated with primary antibodies against pro-collagen type III (1:1,000, monoclonal, clone number: FH-7A, Abcam), collagen type IA1 (1:500, monoclonal, clone number: 3G3, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated IP3 receptor (1:1,000, polyclonal, Cell Signaling Technology, Beverly, MA, USA) and phosphorylated-CaMKII (1:2000, polyclonal, Abcam) and secondary antibodies. Bound antibodies were visualized using the ECL detection system (Millipore, Darmstadt, Germany) and analyzed on AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). β-actin (Sigma) was used as a loading control to confirm equal protein loading and then normalized to the value of control cells.

**Intracellular Ca²⁺ imaging**

Ca²⁺ imaging was conducted as described previously [24]. In brief, cardiac fibroblasts treated with or without 10, 100, 1,000 nmol/L testosterone for 24 hours on a coverslip (1 × 1 cm) were incubated with 5 μmol/L fura-2-acetoxymethyl ester (Life Technologies, Carlsbad, CA, USA) and 2.5 μg/mL Pluronic F-127 (20% solution in dimethyl sulfoxide, Sigma) in a Ca²⁺-free solution with (in mmol/L) KH2PO4 1.2, NaCl 120, MgSO4 1.2, KCl 5.4, HEPES 6, glucose 10 (pH 7.40) for 30 minutes at 36°C with 5% CO2. Fura-2 fluorescence images were taken with a polychrome V monochromator (Till Photonics, Munich, Germany) mounted on an upright Leica DMI 3000B microscope (Leica Microsystems, Buffalo
Grove, IL, USA) with dual excitation wavelengths (340, and 380 nm) and an emission wavelength of 510 nm. MetaFluor software (version 7.7.9.0, Molecular Devices, Sunnyvale, CA, USA) was used for fura-2 image analysis. The ratio of fluorescence from excitation at 340 nm (F_{340}) to F_{380} was used as a marker of the relative level of intracellular Ca^{2+}. To measure Ca^{2+} entry, cells were first exposed to the Ca^{2+}-free solution for 3 minutes followed by the endoplasmic reticulum (ER) Ca-ATPase inhibitor (Thapsigargin, 2.5 μmol/L, Sigma) co-treatment for ER Ca^{2+} store depletion. After the intracellular Ca^{2+} surge from ER Ca^{2+} leak induced by thapsigargin returned to a steady-state, the extracellular Ca^{2+} concentration was increased to 2 mmol/L to measure Ca^{2+} entry through store-operated channels. The change in intracellular Ca^{2+} (ΔF_{340/380}) from the steady-state post-ER Ca^{2+}-induced intracellular Ca^{2+} surge to the plateau state under 2 mmol/L Ca^{2+} solution was used to represent Ca^{2+} entry.

Statistical analysis

All quantitative data are expressed as means ± standard errors of the mean. The paired t-test and one way repeated-measures analysis of variance with post hoc Fisher’s least significant difference test were performed using PASW Statistics (version 18.0, IBM SPSS, Chicago, Illinois, USA) to compare cardiac fibroblasts under different conditions. A p value of <0.05 was considered statistically significant.

Results

NO signal pathway in testosterone-treated cardiac fibroblasts

As illustrated in Fig. 1, physiological testosterone (10 nmol/L)-treated cardiac fibroblasts exhibited higher NO content but lower pro-collagen type I and type III production than testosterone-deficient (control) fibroblasts. However, non-physiological testosterone (100, 1,000 nmol/L)-treated fibroblasts exhibited similar NO and collagen production capabilities as compared to control (testosterone-deficient) fibroblasts. Physiological testosterone not only reduced pro-collagen and phosphorylated CaMKII expression, but also increased phosphorylation at the inhibitory site of IP3 receptor, which could be blocked by L-NAME co-treatment (Fig. 2).

Ca^{2+} signal pathway in testosterone-treated cardiac fibroblasts

In the presence of 2-ABP (50 μmol/L, an IP3 receptor inhibitor), the control and physiological testosterone-treated fibroblasts exhibited similar production of type I, type III collagen, and expression of phosphorylated CaMKII, suggesting that physiological testosterone reduced the profibrotic activities of testosterone-deficient fibroblasts by attenuating the IP3 signaling pathway (Fig. 3). The fura-2 fluorescence image experiments revealed that physiological testosterone-treated fibroblasts exhibited decreased Ca^{2+} entry than testosterone-deficient fibroblasts (Fig. 4). In the presence of KN93 (10 μmol/L, a CaMKII inhibitor), the control and testosterone-treated fibroblasts exhibited similar type I and type III collagen production, suggesting that physiological testosterone reduced the profibrotic activities of cardiac fibroblasts by attenuating the CaMKII signaling pathway (Fig. 5).

Discussion

Physiological testosterone can alleviate cardiac fibrosis in HF rats [15]. In this study, we treated testosterone-deficient fibroblasts (control cells) with non-physiological testosterone (100, and 1,000 nmol/L) and physiological testosterone (10 nmol/L), which is within the range of plasma concentration (8 to 15 nmol/L) of testosterone in male rats [25-30]. We found that physiological testosterone (10 nmol/L) significantly activated NO signaling and reduced collagen production in rat cardiac fibroblasts. NO signaling attenuates the collagen production capability of fibroblasts [22]. Physiological Testosterone attenuates cardiomyocyte senescence by enhancing NO signaling [25]. Moreover, testosterone deficiency led to endothelial dysfunction by reducing NO production [31]. In addition, the anti-fibrotic effects of physiological testosterone can be attenuated using L-NAME, which indicated that testosterone exerts its anti-fibrotic effects through the NO signaling pathway. Differently, non-physiological testosterone (100, and 1,000 nmol/L)-treated fibroblasts exhibited similar collagen and NO production capabilities as compared to control (testosterone-deficient) fibroblasts. Similarly, treatment of physiological testosterone increased endothelial NO synthase (eNOS) activities but administration of non-physiological testosterone reduced eNOS gene expression in endothelial cells [32, 33]. These findings suggest that testosterone at different concentrations may exert dissimilar effects on cardiac fibroblasts. Accordingly, it is critical to provide the optimal (physiological) concentrations of testosterone for the treatment of androgen deficiency in clinical practice.

The Ca^{2+} signaling pathway is the downstream profibrotic signal pathway of multiple cytokines [34, 35]. Ang II induced profibrotic cellular activities in fibroblasts by releasing the Ca^{2+} intracellular store thereby activating the MAPK signaling pathway [36]. Ca^{2+} influx can be initiated through the IP3 signal pathway. Activation of IP3 signaling induces ER Ca^{2+} leak, thereby activating membrane store-operated Ca^{2+} channels such as transient...
receptor potential (TRP) channels and Ca$^{2+}$ entry [37, 38]. Ang II upregulates the interaction between IP3-induced ER Ca$^{2+}$ leak and membrane store-operated Ca$^{2+}$ channels, leading to enhanced fibroblast activities [39]. In the present study, physiological testosterone significantly reduced Ca$^{2+}$ entry. In addition, L-NAME blocked physiological testosterone-activated phosphorylation at the inhibitory site of the IP3 receptor, which indicated that NO, produced by physiological testosterone, may inactivate the IP3-induced Ca$^{2+}$ signal pathway. Protein

![Fig. 1 Effects of physiological and non-physiological testosterone on nitric oxide (NO) production of cardiac fibroblasts. (A) Fluorescence photographs and average data of DAF-2 DA, a marker of NO generation and average data of the fluorimetric dye DAF-FM DA, a marker of intracellular NO levels in testosterone-deficient cardiac fibroblasts (control cells) treated with or without physiological testosterone (10 nmol/L) and non-physiological (100 and 1,000 nmol/L) for 24 hours ($n = 6$ experiments). (B) Examples and averaged data of the expression of pro-collagen type I and type III production in testosterone-deficient (control cells) and testosterone (10, 100, and 1,000 nmol/L)-treated cardiac fibroblasts ($n = 6$ experiments). β-actin was used as a loading control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.]
kinase G, the downstream signaling molecule of the NO/cGMP signal pathway [40], phosphorylates the IP3 receptor thereby inactivating IP3-induced Ca\textsuperscript{2+} signaling [41]. Furthermore, protein kinase G can inactivate TRP channels [42]. Moreover, we found that 2-APB with and without physiological testosterone reduced the profibrotic activities of cardiac fibroblasts to similar degrees. These findings indicated that physiological testosterone reduced fibroblast activities through the regulation of IP3 signaling. However, since there are many types of receptors, pathway, and ion channels involved in intracellular calcium concentration, the finding in this study is only one of the mechanisms of cardiac fibrosis. Modulation of Ca\textsuperscript{2+} entry through TRP channels is also crucial in atrial fibrogenesis or anti-atrial fibrosis therapy [22, 43]. TRP channel inhibitor reduced Ang II-induced collagen production and Ca\textsuperscript{2+} entry in atrial fibroblasts [44].

The Ca\textsuperscript{2+} signal pathway activates the profibrotic signal pathway through the binding of Ca\textsuperscript{2+} and calmodulin-induced CaMKII auto-phosphorylation [45, 46]. Activated CaMKII plays a pivotal role in collagen production in fibroblasts [47]. CaMKII inhibition by KN93 or genetically knocked-down CaMKII can attenuate pathological remodeling-induced cardiac fibrosis [48, 49]. In this study, we found that physiological testosterone reduced phosphorylated CaMKII, which was blocked by L-NAME. In addition, in the presence of 2-APB or KN93, control and physiological testosterone-treated fibroblasts exhibited similar pro-fibrotic activities.

In conclusion, as summarized in Fig. 6, physiological testosterone activates NO production and attenuates the IP3 receptor/Ca\textsuperscript{2+} entry/CaMKII signaling pathway thereby inhibiting the collagen production capability of cardiac fibroblasts.

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Fig. 2  Effects of L-NG-nitro arginine methyl ester (L-NAME), a nitric oxide inhibitor on physiological testosterone-treated cardiac fibroblasts

Examples and averaged data of the expression of pro-collagen type I, and type III, phosphorylation at the inhibitory site of the inositol trisphosphate (pIP3) receptor, and phosphorylation of the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (pCaMKII) in testosterone-deficient (control cells) and testosterone (10 nmol/L)-treated cardiac fibroblasts treated with and without L-NAME (100 μmol/L) for 48 hours (n = 6 experiments). β-actin was used as a loading control. * p < 0.05, ** p < 0.01, *** p < 0.005.
Fig. 3 Effects of 2-aminoethyl diphenylborinate (2-APB), an inositol trisphosphate receptor inhibitor, on physiological testosterone-treated cardiac fibroblasts. Examples and averaged data of the expression of pro-collagen type I, and type III production, and phosphorylated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (pCaMKII) in testosterone-deficient (control cells) and testosterone (10 nmol/L)-treated cardiac fibroblasts treated with and without 2-APB (50 μmol/L) for 48 hours (n = 6 experiments). β-actin was used as a loading control. **p < 0.01, ***p < 0.005.

Fig. 4 Ca\(^{2+}\) entry in physiological testosterone-treated cardiac fibroblasts. Representative intracellular Ca\(^{2+}\) tracing indicating intracellular Ca\(^{2+}\) imaging (left panels) from testosterone-deficient (control), and physiological testosterone-treated (10 nmol/L) cardiac fibroblasts. Cells already been treated for 48 hours, were first incubated with a Ca\(^{2+}\)-free solution for 3 minutes followed by cotreatment with the endoplasmic reticulum (ER) Ca-ATPase inhibitor (thapsigargin, 2.5 μmol/L) for ER Ca\(^{2+}\) store depletion. After the intracellular Ca\(^{2+}\) surge from ER Ca\(^{2+}\) leak induced by thapsigargin was returned to the steady-state, the extracellular Ca\(^{2+}\) concentration was then increased to 2 mmol/L to measure Ca\(^{2+}\) entry through store-operated channels. Ca\(^{2+}\) entry was noted after an increase in extracellular Ca\(^{2+}\) to 2 mmol/L. The right panel shows the average change in intracellular Ca\(^{2+}\) (ΔF\(_{340}/F_{380}\)) from the steady-state post ER Ca\(^{2+}\)-induced intracellular Ca\(^{2+}\) surge to the plateau state under treatment with 2 mmol/L Ca\(^{2+}\) solution of testosterone-deficient (n = 31 from 3 rats) and testosterone-treated (n = 29 from 3 rats) cardiac fibroblasts. Physiological testosterone significantly reduced Ca\(^{2+}\) influx in testosterone-deficient cardiac fibroblasts. ***p < 0.005.
Fig. 5  Effects of KN93, a Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) inhibitor, on physiological testosterone-treated cardiac fibroblasts. Examples and averaged data of the expression of the pro-collagen type I, and type III production in testosterone-deficient (control cells) and testosterone (10 nmol/L)-treated cardiac fibroblasts treated with and without KN93 (10 μmol/L) for 48 hours (n = 5 experiments). β-actin was used as a loading control. * p < 0.05, ** p < 0.01, *** p < 0.005.

Fig. 6  Illustration of the proposed molecular mechanism underlying the anti-fibrotic effects of physiological testosterone on cardiac fibroblasts. Physiological testosterone increases nitric oxide (NO) production, and attenuates the inositol trisphosphate (IP3) receptor signaling pathway thereby reducing Ca\(^{2+}\) entry, phosphorylated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) expression and collagen production in testosterone-deficient cardiac fibroblasts.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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