Androgens Rapidly Activate Nuclear Factor-Kappa B via Intracellular Ca\(^{2+}\) Signalling in Human Vascular Endothelial Cells

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**Abstract**

There exists a striking gender difference in the incidence of atherosclerosis. Androgen exposure may predispose men to earlier onset atherosclerosis. We previously demonstrated that the potent androgen, dihydrotestosterone, enhanced the binding of monocytes to endothelial cells, via androgen receptor/nuclear factor kappa B-dependent expression of the cell adhesion molecules, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. We now show that testosterone and dihydrotestosterone can also induce a novel, non-genomic pathway that leads to the rapid activation of nuclear factor-kappa B via intracellular Ca\(^{2+}\) signalling, initiated at the plasma membrane. Human umbilical vein endothelial cells exposed to 6-60 nM testosterone or dihydrotestosterone showed a rapid increase in intracellular calcium levels. The testosterone or dihydrotestosterone effect on increased intracellular calcium could not be abrogated by pre-incubation with androgen receptor antagonist, hydroxyflutamide, or by U73122, an inhibitor of intracellular calcium release from endoplasmic reticulum stores. However, pre-incubation with both N6\(^{2+}\)or an extracellular Ca\(^{2+}\) chelator blocked the testosterone-induced intracellular Ca\(^{2+}\) surge. Testosterone conjugated to bovine serum albumin was equal to free testosterone in its ability to induce the intracellular Ca\(^{2+}\) surge. Binding studies showed testosterone does bind to the plasma membrane, however, classical androgen receptor was unable to be detected in the plasma membrane of human umbilical vein endothelial cells. Testosterone was found to rapidly increase nuclear factor-kappa B activity, an effect that was blocked when cells were incubated in calcium-free media. This study demonstrates for the first time that testosterone induces a non-genomic membrane-initiated Ca\(^{2+}\) dependent signalling pathway that leads to the rapid activation of nuclear factor-kappa B.

**Keywords:** Androgens; Non-genomic signalling; Calcium signalling; Nuclear factor-kappa B

**Introduction**

Coronary artery disease (CAD) remains a major cause of mortality in the Western world. Epidemiologic studies show that men are twice as likely as women to die of CAD, across a wide variety of populations [1]. While male gender is a well-known risk factor for CAD, the relationship between endogenous and exogenous, pharmacological and physiological doses of androgens and CAD remains unclear. Given the increasing use of androgens in the community for medical treatment as well as anabolic steroid abuse (reviewed in [2]), it is becoming more necessary to understand how androgens influence CAD.

Our laboratory has been investigating the hypothesis that androgens can augment early inflammatory steps in atherogenesis [3,4] as well promote atherosclerotic plaque calcification [5]. We have demonstrated a novel genomic AR/NF-kB mediated pathway for VCAM-1 expression and monocyte adhesion that operates in vascular endothelial cells from male- but not female- donors [6].

The classical pathway of testosterone (T) action involves its binding to a specific receptor, the androgen receptor (AR), a ligand-dependent nuclear transcription factor [7]. However, more recently, it has become apparent that the actions of androgens are more complex with some androgen actions arising through interactions of AR with other transcription factors including NF-kB or non-genomic, fast-acting cell signal pathways that are initiated at the plasma membrane and are not dependent on AR. The existence of a novel plasma membrane receptor for androgens has been described for non-central nervous system tissue, vascular tissue, osteoblasts, macrophages, ovary and T cells [8-15] however, to date, no membrane receptor for androgens has been shown in human vascular endothelial cells. It has been proposed that this membrane receptor stimulates intracellular signalling through interaction with G proteins [16,17] that leads to effects including fast intracellular Ca\(^{2+}\) increases, activation of Ca\(^{2+}\)-dependent enzymes and 2nd messenger cascades [18].

Our previous work identified that the androgen, DHT, increased expression of the pro-inflammatory gene, VCAM-1, via a mechanism that involved activation of the classical IKK/IκBα/NF-κB signalling pathway rather than via an androgen response element (ARE) present in the VCAM-1 promoter region [3]. We now hypothesize that androgens can induce rapid non-genomic Ca\(^{2+}\) signalling that, in turn, activates the classical NF-kB.

**Materials and Methods**

**Reagents and hormones**

Phenol red free-medium 199 (M199) was purchased from Sigma
Chemical Co. (St Louis, MO, USA). Phenol red-free-RPMI was purchased from JRH Biosciences (Brooklyn, Victoria, Australia). Phenol red- and calcium-free media were purchased from Life Technologies (EpiLife; Grand Island, NY, USA). Human serum was obtained from Red Cross Blood Service (Sydney, NSW, Australia) and foetal calf serum (FCS) was purchased from Life Technologies (Carisbad, CA, USA). Serum was charcoal/dextran-removed to strip endogenous steroids. pNF-KB and pRL-TK luciferase reporter vectors were purchased from Promega (Sydney, NSW, Australia). Testosterone, dihydrotestosterone, testosterone 3-(O-carboxymethyl) oxime bovine serum albumin (T-BSA), U-73122, U-73144, nickel (Ni2+) and ethylene glycol-bis(2-aminoethyl) ether-N,N′,N′,N′-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fluo-4 acetoxyethyl ester (Fluo-4 AM) and pluronic-F127 were obtained from Life Technologies. All steroids were dissolved in 95% ethanol and added at a dilution that resulted in a final concentration that never exceeded 0.01% ethanol. This ethanol concentration did not affect [Ca2+]i in any experiment.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described [19]. HUVECs were maintained in M199 supplemented with 20% human serum at 37°C in 5% CO2. HUVECs were used between passages 2-4. Bovine aortic endothelial cells (BAECs) were purchased from Cell Applications (San Diego, CA, USA) and maintained in RPMI supplemented with 10% FCS at 37°C in 5% CO2. BAECs were used between passages 4-12.

[Ca2+]i measurements

HUVECs (1×10⁶) were seeded onto 22-mm glass coverslips and used for the determination of [Ca2+]i within 24 hours. HUVECs were loaded with 3 µM Fluo-4 AM and 0.05% pluronic acid for 30 minutes at room temperature in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 8.4 mM HEPES, adjusted to pH 7.4 with HCl). Cells were then washed twice with HEPES buffer and incubated in HEPES buffer for 15 minutes at room temperature to allow full hydrolysis of the Fluo-4 ester. The coverslips were next attached to a chamber slide mounted onto the stage of a Leica TCS SPII multi-photon inverted phase-contrast microscope (Leica Microsystems, Leidelberg, Germany). HEPES buffer was perfused at slow speed through the chamber with steroids (testosterone 0.6, 6, and 60 nM; dihydrotestosterone 60 nM; extensively dialyzed T-BSA, 60 nM) and reagents (U-73122 2 µM and U-73144 2 µM; Ni2+ 5 mM; EGTA 2 mM) applied locally to cells by pressure ejection via a micropipette.

Calcium measurements were obtained by direct imaging via a real-time fluorescent confocal imaging system. Images were acquired at 488 nm for excitation and 510 nm for emission. The dissociation constant (Kd) for the Fluo-4-Ca²⁺ complex was 345 nM. Maximal fluorescence (Fmax) was determined following cellular exposure to digoxin (25 µM) that allows full interaction between intracellular Fluo-4 and extracellular calcium. Minimal fluorescence (Fmin) was measured using 4 mM EGTA. The cytosolic free calcium concentration was calculated by comparing the emitted cellular fluorescence with the maximal and minimal fluorescence of the cells utilizing the following equation:

\[ [\text{Ca}^{2+}]_i = K_d \frac{[\text{F}-\text{F}_{\text{min}}]}{[\text{F}_{\text{max}}-\text{F}]}. \]

Intracellular free calcium was measured at resting state and following the addition of compounds. Intracellular calcium measurements were averaged from at least 15 cells.

Binding assay

Cells were harvested by cell scraping and ruptured by shear-lysis. The cell homogenates were then loaded onto a 1-22% (w/v) Ficoll gradient with 45% (w/v) Nycozem cushion and ultracentrifuged in a Beckman Ti 65.2 vertical-tube rotor at 30,000 rpm for 90 min at 4°C and 26x200 µL fractions were collected from the bottom of the centrifuge tube. Fractions 15-20 were identified as purified plasma membrane fractions using a number of enzyme assays. These assays were performed on plasma membrane and cell samples both before and after formaldehyde/DTT treatment. The activities of marker enzymes (activity/mg of protein) in the plasma membrane preparation and cell sample post formaldehyde/DTT treatment were quantified as the ratio of activity in plasma membrane over that in whole cell. Aeryl sulfatase was used as a lysosomal marker, succinate dehydrogenase as a mitochondrial marker, galactosyl transferase for Golgi apparatus, glucose-6-phosphatase for endoplasmic reticulum, and alkaline phosphatase and Na·K’-ATPase as plasma membrane markers [20,21]. The binding assay was performed at 4°C using 1 nM [3H]-testosterone (Amersham), 10 µg of the purified HUVEC plasma membrane and binding buffer (50 mM Tris pH 7.4, 5 mM MgCl₂, 1 mM CaCl₂, 0.6 mM EDTA, 0.1% BSA) to a final volume of 250 µL. In parallel samples, an excess of unlabelled testosterone (10 µM) was added to determine any non-specific binding. The binding reaction was incubated for 90 mins. At the end of the incubation period, 50 µL aliquots (X4 of each sample) were rapidly filtered under vacuum through DEAE glass fiber filters (Grade FG/C, Whatman) via a suction manifold. Filters were immediately washed 4 times with 1 mL ice-cold binding buffer under vacuum and air-dried. The filters were placed in vials and extracted with 5 mL Ultima Gold-F liquid scintillation cocktail (Perkin Elmer) in scintillation vials overnight. The radioactivity of bound testosterone retained on the filter was measured using a scintillation counter. Specifically-bound [3H]-testosterone was determined by subtracting the non-specific binding from binding to the membrane incubated only with [3H]-testosterone (total binding).

Whole cell lysates from HUVECs and prostate cancer cells (LNCaP) were used as positive controls for the binding studies.

Western blotting

Total cell protein was extracted using RIPA lysis buffer [1% nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris (pH 8), 1 µl/ml protease cocktail inhibitor (Sigma)]. Plasma membrane (100 µg) and whole cell lysate (20 µg) proteins were resolved on an 8% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% skim milk powder in TBST [250 mM NaCl, 20 mM Tris (pH 7.5), 0.05% Tween 20] for 1 hour, washed with TBST (3x, 5 min) before overnight incubation with mouse monoclonal anti-human AR antibody (1:500 dilution; Santa Cruz). Membranes were then washed in TBST (3x; 5 min) and incubated with HRP-conjugated (1:10,000 dilution; Sigma) secondary antibody for 2 hours. Bound secondary antibody was detected after washing with TBST (3x; 5 min) by an enhanced chemiluminescence (ECL) system (Amersham) and exposure to Hyperfilm (Amersham).

Transfection

BAECs, rather than HUVECs, were used for the luciferase reporter assays to increase transfection efficiency [22]. Twenty-four hours before transfection, BAECs were seeded (1x10⁶ cells/well) onto a 12-well plate in RPMI with 10% FCS. Cells were washed and exposed to a mixture of 600 ng NF-kB-luciferase reporter vector DNA, 120 ng TK-renilla reporter vector DNA and Effectene reagent (Qiagen), as per the
manufacturer’s instructions. Forty-eight hours post-transfection, cells were treated for 7.5, 15 and 30 mins with T (100 nM), DHT (400 nM), T-BSA (100 nM) or 0.1% ethanol (control). Cells were harvested by the addition of 250 µl 1X passive lysis buffer (Promega). Luciferase and renilla luminescent signals were determined by the Dual-Luciferase Reporter assay system (Promega), according to the manufacturer’s protocol. To correct for transfection efficiency, luciferase activity was normalized to renilla activity.

Statistical analysis

Calcium measurements were averaged over at least 15 cells, from 4 different human donors. Results of transfection studies are reported as mean ± SEM compared to controls. Unpaired Student t-tests were used to determine the significance of changes between groups. A value of P < 0.05 was regarded as statistically significant.

Results

T and DHT rapidly increase intracellular Ca²⁺ levels

Non-genomic androgen pathways are characterised by intracellular Ca²⁺ signaling so we first tested whether T or DHT increased intracellular Ca²⁺ levels in HUVECs. Figure 1A showed that T induced a dose-dependent effect at 6 and 60 nM concentrations (both P<0.05) increasing [Ca²⁺], by 100-170 nM. DHT at 60 nM also produced a significant increase in [Ca²⁺], (Figure 1B) but was less effective than T (112 ± 15 nM versus 170 ± 25 nM, respectively) in increasing intracellular Ca²⁺ levels, in keeping with what has been shown in other cell types [22-24]. Vehicle control (ethanol 0.01%) did not alter intracellular Ca²⁺ levels.

T induces Ca²⁺ influx into cells

The androgen-induced increase in [Ca²⁺], could be due to the release of Ca²⁺ from intracellular stores and/or an influx of extracellular Ca²⁺ through plasma membrane channels. To identify if T induced the release of Ca²⁺ from intracellular stores, HUVECs were pre-incubated with the phospholipase C inhibitor, U73122 or its inactive analog, U73343 before activation with 60 nM T. Figure 2A shows that U73122 did not abrogate the T-induced [Ca²⁺]. This was also true if U7344 was used (data not shown). If cells were stimulated with 0.01% ethanol after U73122 treatment there was no Ca²⁺ response. Together, the results suggest that the change in Ca²⁺ response was due to T and not a delayed response to U73122, as this reagent has been reported to directly induce Ca²⁺ release from intracellular stores [25]. To identify if T induced Ca²⁺

influx, HUVECs were pre-incubated with Ca²⁺ channel blocker, Ni²⁺ or extracellular calcium chelator, EGTA before activated with 60 nM T. Figure 2B-C shows that both Ni²⁺ and EGTA effectively blocked the T-induced increase in [Ca²⁺].

T increases Ca²⁺ via plasma membrane signalling

In order to test if the Ca²⁺ signalling response to T originated at the plasma membrane, we next examined whether exposure of HUVECs to T conjugated to BSA increased [Ca²⁺]. T-BSA was dialyzed repeatedly to ensure removal of free T before treating the cells. Figure 3A shows that 60 nM T-BSA effectively increased [Ca²⁺]. BSA (60 nM) had no effect on [Ca²⁺].

The finding that T-BSA increased intracellular Ca²⁺ levels suggested that T activated a receptor present in the plasma membrane. To explore this further, we prepared protein extracts from the plasma membrane of HUVECs then incubated the protein extract with [³H]-T. Figure 3B shows that the plasma membrane fraction showed a 7-fold higher level of T-binding relative to a cytoplasmic protein extract (P<0.05). To determine the relative amount of binding, we measured T-binding to

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a whole cell protein extract from LNCaP prostate cells, an AR positive cell type.

**Classic AR was not detected in the plasma membrane of HUVECs**

It has previously been described for some cell types that the non-genomic effects induced by T are mediated by classic AR located in the plasma membrane. However, using Western blot, we were unable to detect AR in plasma membrane protein extracts of HUVECs isolated from male donors (Figure 3C). Again, we used whole cell lysate from LnCaP cells as a positive control as well as whole cell HUVEC protein extracts.

Androgen action via the AR can be blocked by the AR antagonist, hydroxylutamide (HF). To test if HF could abrogate T effects on inducing intracellular Ca\(^{2+}\) levels, HUVECs were pre-incubated with 6 µM HF before activated with 60 nM T. Figure 3D shows that HF did not abrogate the effect of T on inducing [Ca\(^{2+}\)]\(_i\) in HUVEC.

**T and DHT rapidly activate NF-κB**

Given our findings that T rapidly increases intracellular Ca\(^{2+}\) levels, we tested whether T could rapidly activate the Ca\(^{2+}\)-sensitive, NF-κB, in a non-genomic, AR-independent manner. To perform this experiment, we transfected bovine aortic endothelial cells (BAECs) (HUVECs are much harder to transfect with an efficiency <30%) with an NF-κB-luciferase reporter vector. Transfected BAECs were then exposed to T (60 nM), DHT (400 nM) or T-BSA (400 nM) for 0, 7.5, 15, and 30 mins. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. *P < 0.05 vs. vehicle control/BSA. (A & B) Bovine aortic endothelial cells (BAECs) were transfected with an NF-κB-luciferase reporter vector and then stimulated with T (40 nM), DHT (40 nM) or T-BSA (40 nM) for 0, 7.5, 15, and 30 mins. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. *P < 0.05 vs. vehicle control/BSA. (A & B) Bovine aortic endothelial cells (BAECs) were transfected with an NF-κB-luciferase reporter vector and then stimulated with T (40 nM), DHT (40 nM) or T-BSA (40 nM) for 0, 7.5, 15, and 30 mins. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. *P < 0.05 vs. vehicle control/BSA. (A & B) Bovine aortic endothelial cells (BAECs) were transfected with an NF-κB-luciferase reporter vector and then stimulated with T (40 nM), DHT (40 nM) or T-BSA (40 nM) for 0, 7.5, 15, and 30 mins. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. *P < 0.05 vs. vehicle control/BSA. (A & B) Bovine aortic endothelial cells (BAECs) were transfected with an NF-κB-luciferase reporter vector and then stimulated with T (40 nM), DHT (40 nM) or T-BSA (40 nM) for 0, 7.5, 15, and 30 mins. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. Results are presented as percentage of 0 min values and are the mean ± SEM of 3 independent experiments. *P < 0.05 vs. vehicle control/BSA. **Figure 4: Testosterone rapidly induces NF-κB DNA binding activity in endothelial cells.**

**Discussion**

The primary aim of this study was to elucidate whether androgens could elicit fast-acting, non-genomic effects on NF-κB activation in HUVECs. We describe a novel membrane-initiated, AR-independent mechanism mediated by intracellular Ca\(^{2+}\) signalling that ultimately induces the transcriptional activity of the important cell regulator, NF-κB.
NF-κB is a transcription factor that plays a critical role in the coordination of the inflammatory response and cell survival pathways. We have previously reported that a 48 hour exposure of HUVECs to DHT activates NF-κB and subsequently induces monocyte adhesion to endothelial cells, a key early step in atherogenesis [3]. The enhanced monocyte adhesion was due to the DHT effect on increasing cell adhesion molecule expression, namely VCAM-1, on the cell surface on HUVECs. Collectively, our findings now suggest that androgen exposure first activates a non-genomic AR-independent intracellular Ca²⁺ signalling pathway that drives rapid NF-κB activation that is later enhanced by genomic, AR-dependent activation of NF-κB. Our previous studies showed that 12 hours was needed to induce the significant genomic effect. In the present work, there was no attempt to identify the type or identity of the Ca²⁺ channel responsible for T-induced Ca²⁺ influx into HUVECs. There are many different types of calcium channels, both voltage and ligand-gated. In T cells, membrane androgen receptor mediates ligand-induced Ca²⁺ influx through non-voltage-gated, Ni²⁺-blockable Ca²⁺ channels [23,24]. In osteoblasts, T stimulates voltage-gated Ca²⁺ channels and intracellular store release to increase cellular Ca²⁺ levels [15]. Androgens induce vasorelaxation through the inactivation of L-type voltage-operated Ca²⁺ channels and/ or the activation of voltage-operated and Ca²⁺-activated K⁺ channels in smooth muscle cells [26]. Therefore, it could be that T and other androgens bind directly to a ligand-gated Ca²⁺ channel in HUVEC to initiate non-genomic signalling. This now needs to be explored. Of interest is that the vasodilatory effect is structurally specific in a manner that appears to be different to that of non-genomic effects. For example, 5α-DHT exhibits strong genomic effects through the AR, however only shows moderate vasorelaxing activity, whereas its isomer 5β-DHT has weak genomic effects but strong vasorelaxing activity [27-29]. We also observed that DHT is not as effective as T in eliciting a calcium response in HUVEC indicating this same structural specificity for the non-genomic effects.

Non-genomic pathways of androgen action have been previously described for a number of cell types, including osteoblasts [15], macrophages [9,10,30], T cells [23,24], kidney cells [31], granulosa, luteinizing cells [32], Sertoli cells [33], cardiac myocytes [34], skeletal muscle [12], HUVECs [35], among others. For HUVECs, androgens were shown to induce Ca²⁺ levels that were associated with apoptosis [35]. This is in keeping with our findings that T and DHT through both non-genomic and genomic signalling activate NF-κB, a key regulator of inflammatory and apoptosis pathways. For some other cell types, AR-dependent non-genomic signalling has been described however, for HUVECs, we could find no evidence that AR was involved in the rapid response. To date, an estrogen membrane receptor coupled to a G-protein has been identified as GPR30 [36] and a membrane progesterone receptor has been successfully cloned [37]. There is similar evidence for a membrane-bound receptor coupled to a G-protein detected in BAECs [38], skeletal muscle cells [12], glioma (G6) cells [39], cardiac myocytes [34] and LnCaP cells [13,40], although this putative receptor has yet to be cloned.

In summary, our data support the existence of a novel membrane-associated receptor in human vascular endothelial cells that mediates rapid responses to androgens. Furthermore, the data demonstrates that androgens can rapidly activate NF-κB via effects on intracellular Ca²⁺ signalling. A hypothetical model illustrating the induction of NF-κB by androgens through the non-genomic and genomic signalling is depicted in Figure 5. Our findings that androgens can act through non-genomic signalling to activate NF-κB may have important implications for reported vascular effects arising from the administration of pharmacologic exogenous androgens. In cardiac cells, T elicits voltage-dependent Ca²⁺ oscillations and inositol 1,4,5-triphosphate (IP3) receptor-mediated Ca²⁺ release from internal stores, leading to activation of mitogen-activated kinase and mammalian target of rapamycin (mTOR) signalling that promotes cardiac hypertrophy. In concert, with the adverse activation of endothelial cells that we now report, it may be that the adverse health effects observed with androgen abuse such as higher incidence of vascular reactivity and premature cardiovascular complications are due to these non-genomic activation of damage at the cellular level of the vasculature inclusive of enhanced inflammatory processes.

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