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Androgen Hormone Inhibits Expression of iNOS and COX-2 Protein in Rat Vascular Smooth Muscle Cell

Hwa Young Bae[1], Ji Eun Park, Eun Mi Jeon, Young Jin Kang, Kwang Youn Lee, and Hyoung Chul Choi

Department of Pharmacology, College of Medicine, Yeungnam University, Daegu 705-717, [1]Department of Pediatrics, Ulsan University Hospital, College of Medicine, University of Ulsan, Ulsan 682-060, Korea

We investigated the effects of testosterone and dihydrotestosterone on inflammatory response of iNOS and COX-2 expression in rat vascular smooth muscle cells. Rat vascular smooth muscle cells (VSMC) stimulated with bacterial lipopolysaccharide (LPS; 10 μg/ml) for 24 hours were incubated with increasing amounts of testosterone and dihydrotestosterone (1 and 100 nM). LPS was found to induce inflammatory response of iNOS and COX-2 mRNA and protein in VSMC. These processes were affected by male sex steroid hormones. For 3 hours, however, pretreatment of the cells with 100 nM each of testosterone and dihydrotestosterone suppressed LPS induced iNOS and COX-2 protein expression. RT-PCR analysis revealed that testosterone and dihydrotestosterone did not inhibit mRNA expression of iNOS and COX-2 stimulated by 24 hours of LPS incubation. Proliferation rate was slower in VSMC treated with testosterone and dihydrotestosterone. Testosterone enhanced androgen receptor expression, and LPS significantly reduced androgen receptor protein expression in VSMC. These results indicate that the expression of both iNOS and COX-2 proteins was suppressed by testosterone and dihydrotestosterone in LPS stimulated VSMC and leading to reduction of vascular inflammation.

Key Words: iNOS, COX-2, Testosterone, Dihydrotestosterone, VSMC

INTRODUCTION

Gender differences in cardiovascular disease have been attributed to hormonal differences between women and men (Wenger et al, 1993). There is considerable interest on the effects of estrogen on the vasculature (Hulley et al, 1998; Rossouw et al, 1998), however, the interaction between androgens and cardiovascular function and disease has attracted much less attention (Teoh et al, 2000).

Although recent data from clinical trials do not support the purported cardiovascular effects of testosterone hormonal therapy (Tenover, 1999), there is substantial evidence on the beneficial cardiovascular effects of androgens, such as vasodilation (Yue et al, 1995; Ding & Stallone, 2001) and inhibition of vascular smooth muscle cell (VSMC) proliferation (Williams et al, 1995).

Inflammation and death of VSMC are important mechanisms in vascular disease and remodeling such as atherosclerosis (Barzilay & Freedland, 2003). Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are important inflammatory markers of VSMC, which are predominantly expressed in cardiovascular disease (Choi et al, 2002; Egan et al, 2004). Furthermore, recent studies have shown that COX-2 is expressed by endothelial cells, VSMC, and macrophages in atherosclerotic lesions (Schonbeck et al, 1999; Stemme et al, 2000). Several lines of evidence suggest an effect of androgen on these processes (Hutchison et al, 1997; Hashimura et al, 2005; Phillips, 2005). Inhibition of iNOS expression by several steroids hormone, including glucocorticoid and aldosterone, has been described (Di Rosa et al, 1990; Ikeda et al, 1995).

Several studies indicate that the cardiovascular system is a target for direct effects of androgen hormone. Testosterone, the main male sex hormone, has been shown to influence the release of endothelium derived relaxing factor (Valenti et al, 2003; Sainz et al, 2004), implying an inhibition of constitutive nitric oxide synthesis by testosterone. Nevertheless, no definite data are known to show an influence of male sex hormone on the inflammatory mediator, iNOS and COX-2.

Although evidence suggests that hypogonadal males actually have an increased rate of cardiovascular events (Hak et al, 2002), the effects of androgen on vascular inflammation remain to be fully elucidated, and the mechanism by which androgen modulates vascular inflammation remains unclear.

In the present study, therefore, we investigated whether testosterone and dihydrotestosterone affect the expression of iNOS and COX-2 in VSMC after LPS stimulation and its precise mechanism using transcription factor, NFκB p65 and IκB-α. We also examined male sex steroid hormone receptor, androgen receptor (AR), expression in vascular inflammation.

Corresponding to: Hyoung Chul Choi, Department of Pharmacology, College of Medicine, Yeungnam University, 317-1 Daemyeong 5-dong, Nam-gu, Daegu 705-717, Korea. (Tel) 82-53-620-4353, (Fax) 82-53-656-7995 (E-mail) hcchoi@med.yu.ac.kr

ABBREVIATIONS: iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; VSMC, vascular smooth muscle cell; AR, androgen receptor; LPS, lipopolysaccharide.

METHODS

Cell culture

Rat aortic VSMC was derived from thoracic aorta of Sprague Dawley adult male rat by primary explant culture techniques. Briefly, excised thoracic aortas were minced into small pieces and washed by HBSS (Sigma, St. Louis, USA). These pieces were plated onto a culture dish containing 50% fetal bovine serum (FBS) (Gibco BRL, Grand Island, USA) and antibiotics-antimycotics (penicillin 100 U/ml, amphotericin 2.5 μg/ml and streptomycin 100 μg/ml) in DMEM (Sigma, St. Louis, USA). Outgrownd cells were plated and grown in DMEM supplemented with 10% FBS and antibiotics-antimycotics. Cells were maintained in a humidified 95% O_2-5% CO_2 incubator. Media were changed two or three times a week.

Western blot

Confluent VSMC grown in media supplemented with 10% FBS were treated with LPS. After stimulation, cells were washed with phosphate buffered saline (PBS) and harvested by scraping. Protein extracts (12,000×g supernatant) in the lysis buffer (20 mM Tris, pH 7.5, EDTA 0.5 mM, EGTA 0.5 mM, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μl/ml β-mercaptoethanol) were resolved by electrophoresis (80 μg protein per lane) on 10% polyacrylamide gels in the presence of sodium dodecylsulphonate and then transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany). The loading and transfer of equal amounts of protein in each lane were verified by staining the protein bands with Ponceau S (0.2% in 3% trichloroacetic acid). After extensive washing with distilled water to remove the protein stain, blots were blocked with 5% nonfat dry milk in PBS. The immobilized protein was visualized by subsequent incubation with a primary antibody (iNOS, COX-2, NF κB p65, I κB-α, androgen receptor antibody) according to each experiments and a secondary polyclonal peroxidase-conjugated anti-rabbit antibody, followed by staining with the enhanced chemiluminescence (ECL) technique developed by NEN life science.

Measurement of cell proliferation

The MTT assay, based on the method of (Oka et al, 1992), measures the conversion of 3-(4,5-dimethylthiazol-2yl)-2, 5-biphenyl tetrazolium bromide to an insoluble formazan precipitate by mitochondrial dehydrogenases present only in viable cells. Twenty microliters of MTT solution were added to 200 μl medium in each well of the 96-well plate, and the plate was incubated at 37°C for 4 hours. The medium was then removed by aspiration, 20 μl of 1× trypsin/EDTA were added to each well to detach the cells, and the plate was placed on a plate shaker for 5 min at room temperature, to facilitate the release tetrazolium salt. Finally, 100 μl of isopropanol/HCl per well were added, the plate was shaken for further 30 min, and the absorbance at OD 590 nm was measured using the microplate reader.

Reverse transcriptation-polymerase chain reaction

Total cellular RNA was isolated using RNAzol B (Tel-Test Inc., Friendswood, USA). RNA concentrations were calculated from absorbance at 260 and 280 nm. For reverse transcriptation, the following components were added to the reaction vials: 5 μg of total RNA, (both sense and antisense primer), 10× first-strand buffer, RNase inhibitor, dNTPs, and MMLV-RT in a total volume of 25 μl. The vials were incubated for 60 min at 37°C, thereafter, the reverse transcriptation was terminated by heating at 95°C for 5 min. The PCR was carried out with 5 μl of RT reaction mixtures, 10× Taq DNA polymerase buffer, dNTPs, both sense and antisense primers, Taq DNA polymerase, and DEPC-treated water in a total volume of 25 μl. The samples were placed in a GeneAmp PCR system 2,400 (Perkin Elmer, Shelton, USA) which was programmed as follows: Pre-PCR; 94°C 1 min, PCR (30 cycle) denaturation; 94°C 1 min, annealing; 52°C 1 min, extension; 72°C 1 min, Post-PCR; 72°C 10 min. The PCR products (10 μl) were size-fractionated by agarose (1.5%) gel electrophoresis, stained with ethidium bromide and visuallized by the use of an ultraviolet transilluminator. The sequence of the two androgen receptor-specific primers was 5-CCCAGGAATTC-CTGTGCATGAAAGC-3 (sense) and 5-CCCCAAGGCACT-GCAGAGAAGTAGT-3 (antisense), and the predominant cDNA amplification product was predicted to be 458 bp in length. The sequence of the two iNOS-specific primers was 5-ATGCCTTGCCCCTGGAAGTTT CTC-3 (sense) and 5-CCTCTGATGGTGCCATCGGGCATCTG-3 (antisense), and the predominant cDNA amplification product was predicted to be 800 bp in length. The sequence of the two COX-2-specific primers was 5-ACACTCTATCACTGGCATCC-3 (sense) and 5-GAAGGGACACCCTTTCACAT-3 (antisense), and the predominant cDNA amplification product was predicted to be 584 bp in length. The sequence of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers was 5-GTC ATGAGCCCTTCCACGATGC-3 (sense) and 5-AATC-TACTGGCGTCTTTCACC-3 (antisense), and predominant cDNA amplification product was predicted to be 300 bp in length. RT-PCR of GAPDH served as a positive control.

Statistical analysis

Results are reported as mean±S.E.M. Comparisons of the means of the two groups were performed by the paired t test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Effects of androgen on iNOS and COX-2 protein expression

VSMC was pretreated with testosterone and dihydrotestosterone for 3 hours, and it was then activated with 10 μg/ml LPS for 24 hours. As shown in Fig. 1A, testosterone dose-dependently decreased the expression of iNOS and COX-2 protein in VSMC induced by LPS. Dihydrotestosterone similarly, although somewhat smaller, inhibited the iNOS and COX-2 protein expression (Fig. 1B). Testosterone (100 nM) induced COX-2 protein without LPS treatment (Fig. 1A). GAPDH levels remained unchanged during incubation with testosterone or dihydrotestosterone. Densitometric analysis of androgen effect on the iNOS and COX-2 protein expression is shown in Fig. 2.

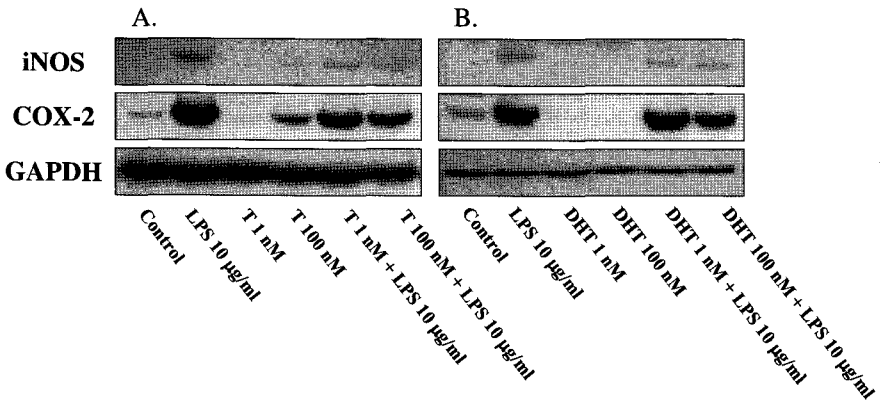

Fig. 1. The effects of testosterone and dihydrotestosterone on LPS induced iNOS and COX-2 protein expression. Vascular smooth muscle cells were pretreated for 3 hours with indicated concentrations of testosterone (A) or dihydrotestosterone (B), then stimulated with LPS for 24 hours. There was an increase in the density of bands on immunoblots, corresponding to iNOS and COX-2 protein after LPS stimulation. GAPDH was used as an internal control. LPS: lipopolysaccharide, T: testosterone, DHT: dihydrotestosterone.

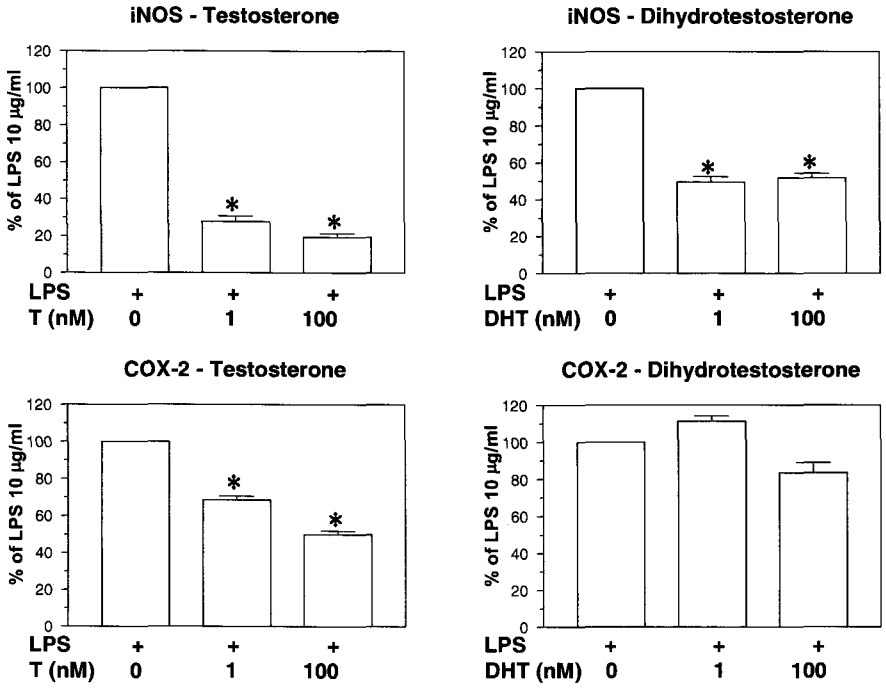

Fig. 2. Densitometric analysis of testosterone and dihydrotestosterone effect on LPS induced iNOS and COX-2 protein expression. Vascular smooth muscle cells were pretreated for 3 hours with indicated concentrations of testosterone or dihydrotestosterone, then stimulated with LPS for 24 hours. LPS: lipopolysaccharide, T: testosterone, DHT: dihydrotestosterone. *$p < 0.05$, compared with cells LPS alone treatment.

Effects of androgen on VSMC proliferation

To investigate anti-proliferative effects of testosterone and dihydrotestosterone, MTT assay was carried out. Drug treatment protocol was the same as mentioned above. Slower proliferation occurred in VSMC when treated with testosterone and dihydrotestosterone. In cells cultured in media with LPS, the results were almost the same as in the presence of testosterone and dihydrotestosterone alone medium (Fig. 3).

Mechanism of androgen action in VSMC inflammation

Western blotting analysis showed that tyrosine kinase, assessed by NF κB p65 and I κB-α protein, was activated

by LPS stimulation for 24 hours with or without androgen pretreatment. Activation of NF κB p65 was increased by testosterone, compared with dihydrotestosterone. I κB-α expression was not significantly changed by dihydrotestosterone pretratment (Fig. 4).

Androgen receptor modulation by VSMC inflammation

Interestingly, expression of the androgen receptor protein in VSMC was dose-dependently increased by 24 hours of testosterone treatment. However, LPS suppressed testosterone induced increase of androgen receptor expression (Fig. 5A).

Dihydrotestosterone did not influence androgen receptor expression in VSMC during 24 hours of incubation. Similary, LPS did not suppress androgen receptor protein expression induced by dihydrotestosterone pretreatment (Fig. 5B).

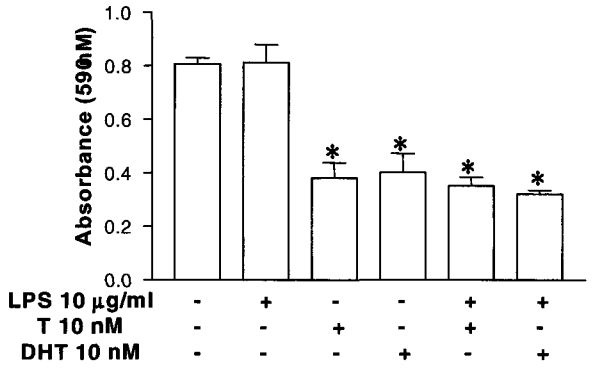

Fig. 3. The effects of testosterone and dihydrotestosterone on LPS induced changes in cell proliferation. Vascular smooth muscle cells were pretreated for 3 hours with indicated concentrations of testosterone or dihydrotestosterone, then stimulated with LPS for 24 hours. Cell proliferation was determined 24 hours after LPS stimulation by using the MTT colorimetric assay. LPS: lipopolysaccharide, T: testosterone, DHT: dihydrotestosterone. *$p<0.05$, compared with cells without any treatment (Control).

Effects of androgen on androgen receptor, iNOS, and COX-2 mRNA expression

VSMC was pretreated with testosterone and dihydrotestosterone for 3 hours, and then activated with 10 μg/ml LPS for 24 hours. As shown in Fig. 6, testosterone and dihydrotestosterone did not suppress androgen receptor, iNOS, and COX-2 mRNA levels in VSMC, although GAPDH level remained unchanged during incubation with testosterone or dihydrotestosterone.

DISCUSSION

iNOS is most readily observed in vascular wall of patients with septic shock or multiple organ dysfunction syndrome (Miki et al, 2005), and has been observed in many cells, including VSMC (Choi & Lee, 2004), endothelial cells (Di Napoli et al, 2005), and macrophage (Shimomura-Shimizu et al, 2005). It generates a large amount of nitric oxide upon stimulation over a prolonged period of time.

COX-2 is an inducible isoform of cyclooxygenase, whose expression is initiated by inflammatory process (Vane, 1994). COX-2 is expressed in many cell types and has a variety of different signal transduction roles. In addition to its role in the inflammation process, it has also been shown to regulate proliferation of many cell types (Cao & Prescott, 2004; Hasegawa et al, 2005).

After vascular inflammation, such as LPS or TNF α stimulation, diverse signalling mechanisms became activated in VSMC, leading to iNOS and COX-2 expression. There is a growing concern and indeed evidence to show that premature mortality due to cardiovascular diseases is promoted by inflammatory mechanisms (Festa & Haffner, 2005). We recently showed that the modulation of LPS signalling is important in VSMC proliferation and survival in septic shock (Choi et al, 2001). Increment of iNOS and COX-2 expression has been found in several vascular inflammatory diseases (Niederberger et al, 2004; Nachtigal

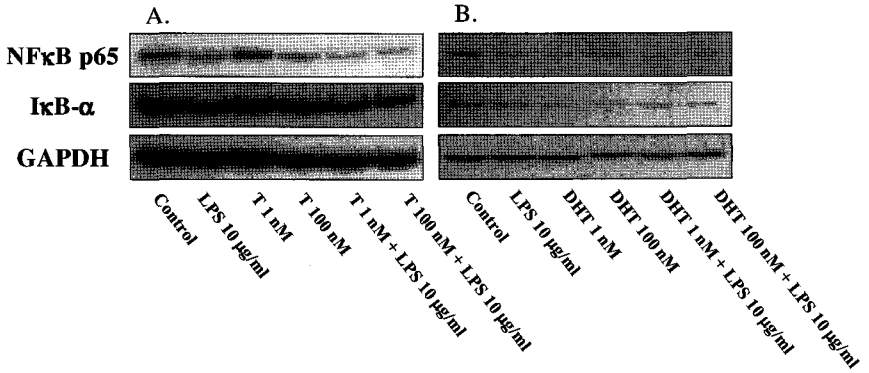

Fig. 4. The effects of testosterone and dihydrotestosterone on LPS induced NF κB p65 and I κB-α protein expression. Vascular smooth muscle cells were pretreated for 3 hours with indicated concentrations of testosterone (A) or dihydrotestosterone (B), then stimulated with LPS for 24 hours. There was an increase in the density of bands on immunoblots corresponding to NF κB p65 and I κB-α protein after LPS stimulation. GAPDH was used as an internal control. LPS: lipopolysaccharide, T: testosterone, DHT: dihydrotestosterone.

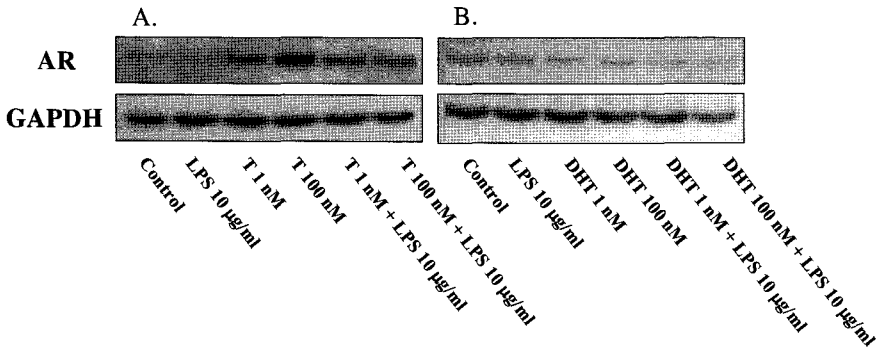

Fig. 5. The effects of LPS on testosterone and dihydrotestosterone induced androgen receptor protein expression. Vascular smooth muscle cells were pretreated for 3 hours with indicated concentrations of testosterone (A) or dihydrotestosterone (B), then stimulated with LPS for 24 hours. There was a change in the density of bands on immunoblots, corresponding to androgen receptor protein after testosterone treatment. GAPDH was used as an internal control. LPS: lipopolysaccharide, T: testosterone, DHT: dihydrotestosterone.

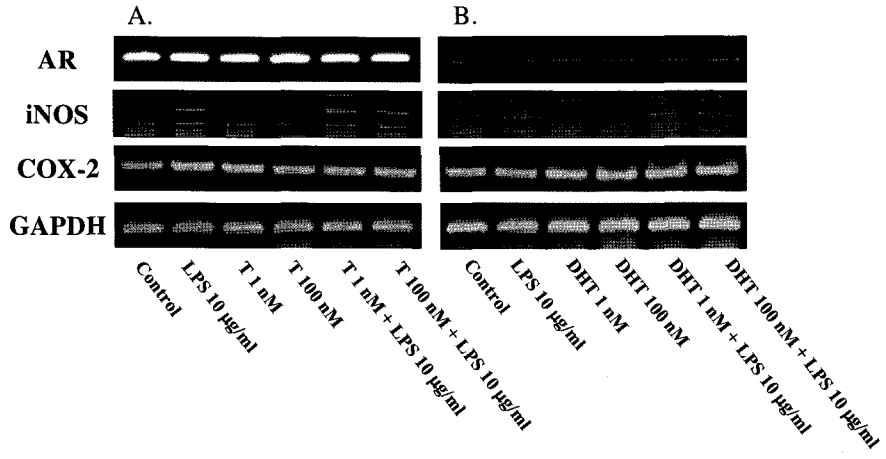

Fig. 6. The effects of testosterone and dihydrotestosterone on androgen receptor and LPS induced iNOS and COX-2 mRNA expression. Vascular smooth muscle cells were pretreated for 3 hours with indicated concentrations of testosterone (A) or dihydrotestosterone (B), then stimulated with LPS for 24 hours. GAPDH was used as an internal control. LPS: lipopolysaccharide, T: testosterone, DHT: dihydrotestosterone.

et al, 2005). The NFκB/IκB dissociation in particular has recently received an interest, because of its role in LPS induced vascular inflammation (Ahn et al, 2005; Vo et al, 2005).

Testosterone is known to act as a transcription factor, when bound to the androgen receptor. Recently, the existence of androgen receptors on VSMC has been described (Hanke et al, 2001). The physiological plasma level of testosterone in males (free and bound testosterone) reaches to 40 nm (according to testosterone plasma levels of up to 10 ng/ml), and it is the principal androgen acting in tissues lacking 5α-reductase. In tissues expressing 5α-reductase, testosterone is converted to dihydrotestosterone, and dihydrotestosterone in these tissues is principal androgen (Marks et al, 1999). The effects of testosterone that are not mediated by dihydrotestosterone are generally considered to be desirable, and they include increased muscle mass and bone density (Matsumoto et al, 2002). However, the effects of dihydrotestosterone appear to be undesirable in such cases, including acne, male pattern baldness, and benign prostate hyperplasia.

In our present experiment, 1 and 100 nM of androgen concentration dissolved in DMSO were used. Ethanol, the most appropriate solvent for testosterone, is known to be a potent inbihitor of iNOS, therefore, had to be avoided (Greenberg et al, 1998).

In the present study, we demonstrated a dose-dependent decrement of iNOS and COX-2 protein expression in LPS stimulated VSMC by treatment with increasing amounts of testosterone or dihydrotestosterone (1 and 100 nM) for 24 hours. When activated cells were pretreated with 100 nM testosterone, iNOS expression was reduced to 20%, although COX-2 expression was inhibited by nearly 50%. However, testosterone and dihydrotestosterone did not reduce iNOS and COX-2 mRNA levels under our experimental condition, because iNOS and COX-2 mRNA expressions are expected to saturate during 24 hours of LPS stimualtion.

The treatment of androgen was found to significantly reduce VSMC proliferation determined by MTT assay. LPS showed no effect on VSMC proliferation, compared with controls. However, testosterone and dihydrotestosterone reduced proliferation of VSMC at 24 hours of incubation. Collectively, these data suggest that the effect of androgen on VSMC proliferation is independent of LPS.

NF κB is known to be indispensable for LPS induced expression of both iNOS and COX-2. It is, therefore, highly likely that the inhibition of LPS induced NF κB activation leads to the supression of both iNOS and COX-2 expression (Shimomura-Shimizu et al, 2005). In the presence of testosterone, LPS induced activation of NF κB p65 was inhibited. The inhibition of LPS induced iNOS and COX-2 expression by testosterone was probably due to the influence on NF κB p65 activation, since I κB-α degradation was not noted, but rather a marked decrease of NF κB p65 expression.

Furthermore, the importance of androgen receptor in vascular inflammatory process was indicated by the finding that testosterone enhanced androgen receptor expression and LPS significantly reduced androgen receptor protein expression in VSMC.

While testosterone and dihydrotestosterone mediate separate effects in different tissues, there is a strong evidence to show that they act through the same receptors. In VSMC, testosterone is a main male sex hormone, because VSMC lacks of 5 α-reductase.

In conclusion, the current data demonstrate that expression of both iNOS and COX-2 was reduced by testosterone and dihydrotestosterone in LPS stimulated VSMC. Our results also suggest that testosterone affects the NF κB signalling pathway, leading to an inhibition of vascular inflammation. In this regard, it seems to be of interest to investigate changes of androgen receptor expression by androgen as an anti-inflammatory therapy.

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