Medical Journal of the
Islamic Republic of Iran

Volume 13
Number 3
Fall 1378
November 1999

FIVE ALPHA DIHYDROTESTOSTERONE (5α-DHT) MAY MODULATE NITRIC OXIDE RELEASE VIA ENDOGENOUS CYTOKINES IN PERITONEAL MACROPHAGES OF NZB/BALBc MICE

KAZEM AHMADI-RENANI AND ALAN B. McCRUDEN*

*From the Department of Immunology, Faculty of Medicine, Baghiatollah University of Medical Sciences, Tehran, 19568, I.R. Iran, and the *Department of Immunology, University of Strathclyde, Glasgow, G4 ONR, U.K.*

ABSTRACT

Recent studies have established that sex hormones directly or indirectly affect T and B cells and macrophages by manipulating the production of cytokines. In this study the possibility of the effect of 5α-DHT on macrophage (Mϕ) nitric oxide (NO) release via interleukin-1, 6 (IL-1β, IL-6) or tumor necrosis factor-α (TNF-α) was investigated. The endogenous cytokines IL-1β, IL-6 and TNF-α were neutralized by hamster anti-mouse IL-1β, rat anti-mouse IL-6 or goat anti-mouse TNF-α monoclonal antibody, respectively. Blocking of IL-1β and TNF-α resulted in decrease in NO release. Neutralizing of IL-6 caused an increase in nitric oxide (NO) production. With regard to these findings, it can be concluded that 5α-DHT may enhance NO production in peritoneal macrophages via modulation of cytokine secretion.

MJIRI, Vol. 13, No. 3, 207-211, 1999
Keywords: Nitric oxide, Cytokine, Testosterone.

INTRODUCTION

Sex steroid hormones have profound effects on the immune response. The effect of sex hormones on the production of cytokines has been extensively studied.[1-5] Among the cytokines influenced by steroid hormones, IL-1, IL-6, TNF, gamma-interferon (γ-IFN) and IL- 4 have been the most extensively studied. The effect of steroid hormones on cytokine production, both in peritoneal macrophages and blood mononuclear cells show similar results. Since macrophages secrete important immune-related substances (IL-1, IL-6, IL-8, TGF-β, NO, etc.), thus, the most probable way steroid hormones could influence the immune response via macrophages would be by modulation of these immune regulatory factors.[5-9]

Neutralizing antibodies against induced cytokines can be used to determine whether they block a given effect. In whole organisms, however, such investigations are rarely possible because of the difficulty of completely blocking production or activity of an induced protein.

Sometimes a cytokine may not induce the synthesis of another cytokine on its own but can 'prime' cells to become responsive to a coinducer. For example, γ-IFN can prime monocytes and macrophages (mϕ) to produce TNF-α or β when stimulated with lipopolysaccharide (LPS) or IL-2.

Interleukin - 4 (IL-4) was the first mediator recognized to be involved in the natural mechanisms for regulation of NO synthesis.[10] The inhibitory effect of IL-4 on NO production was later supported by Leal,[11] who demonstrated that macrophages pre-incubated with IL-4 before activation with LPS and γ–IFN are unable to kill leishmania. Pretreating the cells with IL-10 has also been reported to significantly

Table I. The effect of different concentrations of 5α-DHT on NO_2^- release by activated peritoneal Mϕ of male and female NZB/BALBc mice.

No.	5α-DHT/M	Male (NO_2^-/nM/well)	Female (NO_2^-/nM/well)
1	Control	45.2	49.4
2	1×10^{-13}	44.9	50.3
3	1×10^{-12}	45	50.9
4	1×10^{-11}	45.2	51.5
5	1×10^{-10}	48.1	54.3
6	1×10^{-9}	56.5	61.2
7	5×10^{-9}	63	61.3
8	1×10^{-8}	61	64
9	1×10^{-7}	61.2	65.5
10	1×10^{-6}	63.3	64.2

inhibit NO expression.[12] Another cytokine, transforming growth factor-β (TGF-β), can also inhibit NO synthesis. In contrast, migration inhibitory factor (MIF) has been shown to activate macrophages to produce NO.[14]

MATERIALS AND METHODS

Male and female NZB/BALBc mice (8 weeks old) were sacrificed by cervical dislocation. The peritoneal cells were extracted and were plated out as previously described.[6] After washing the non-adherent cells, remaining cells were then cultured in 0.5 mL complete phenol red free medium (RPMI 1640) containing 0.15 mM l-arginine. Adherent cells (Mϕ) were then activated with 10 μg/mL lipopolysaccharide (LPS) (Sigma) and 100 U/mL γ-IFN. Various concentrations of 5α-DHT (Sigma) in the range of 1×10^{-6}M to 1×10^{-13}M were added and cells incubated at 37°C at 5% CO_2 in air up to 72 h. In some experiments cells were treated with above concentrations of 5α-DHT without stimulating with LPS or γ-IFN. Culture medium was removed from each well, placed in 1.5 mL plastic Eppendorf tubes and centrifuged at 13000g for 10 minutes at room temperature. The supernatants from the centrifuged tubes were transferred to clean tubes and stored at -20°C until analyzed for nitrite.

Regulation of nitric oxide production through cytokines

Peritoneal macrophages were extracted as previously described,[9] plated out and incubated at 37°C for 2 h at 5% CO_2 in air. Non-adherent cells were removed and fresh phenol red free complete medium containing 10 μg/mL LPS, 0.15 mM L-arginine, 15 mM HEPES was added at 0.5 mL. In this experiment, in order to ascertain whether endogenous cytokines released by macrophages had any effect on NO production, IL-1β, IL-6 and TNF-α release were blocked by the addition of appropriate Ab. Briefly, 10 μg/mL specific Ab for each of the above cytokines was added and cells were incubated for a futher 24h.[15] Culture medium was removed from each well and stored as above.

Nitrite assay

Samples were thawed at room temperature. Nitrite concentration in the supernatant was measured using a microplate assay based on the method of Green.[16] 50 or 100 μL aliquots were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphtylendiamine dihydrochloride (Sigma) and 2.5% H_3PO_4) and incubated at room temperature for 10 minutes to form a chromophor. After 10 minutes absorbance was measured at 540 nm in a Multiscan Titertek MCC/340. NO_2^- was determined using sodium nitrite ($NaNO_2$) as a standard. The concentration of NO_2^- obtained in the experiments was corrected by subtracting of NO_2^- residing obtained using fresh medium alone. The detection of nitrite was used as an indicator of NO.

RESULTS

Incubation of activated peritoneal Mϕ with 5α-DHT (1×10^{-6}M to 1×10^{-13}M) for 24 h induced a concentration-

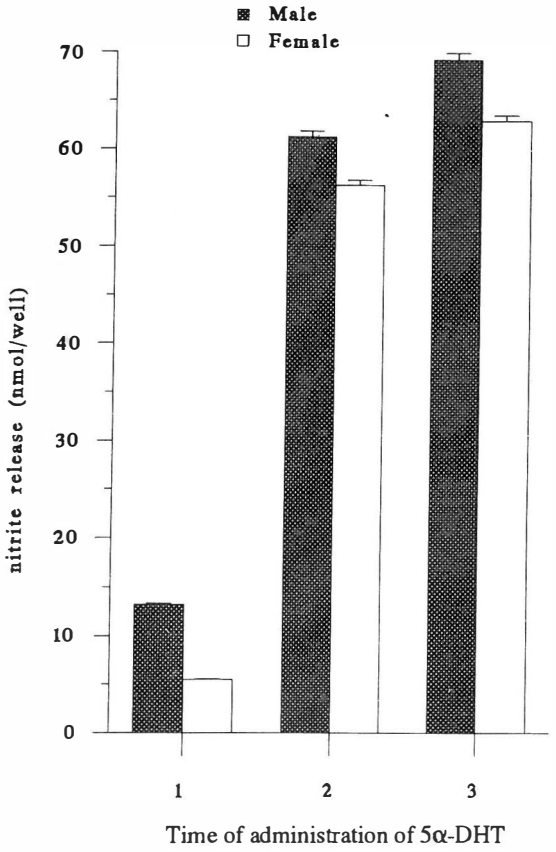

Fig. 1. The effect of time of administration of 5α -DHT on NO_2^- release. (1) Male or female NZB/BALBc peritoneal macrophages were treated with 5 nM 5α -DHT without LPS &γ-IFN and incubated for 24 h. (2) Macrophages were treated with 5nM 5α-DHT at zero time and incubated for 1h, after which LPS (10 μg/mL) and γ-IFN (100 U/mL) were added and cells were incubated for a further 24 h. (3) Macrophages were treated with 5 nM 5α-DHT concurrent with LPS (10 μg/mL) and γ-IFN (100 U/mL) and cells incubated for 24 h. NO_2^- was measured at 24 h after zero time. n=5, T- test.

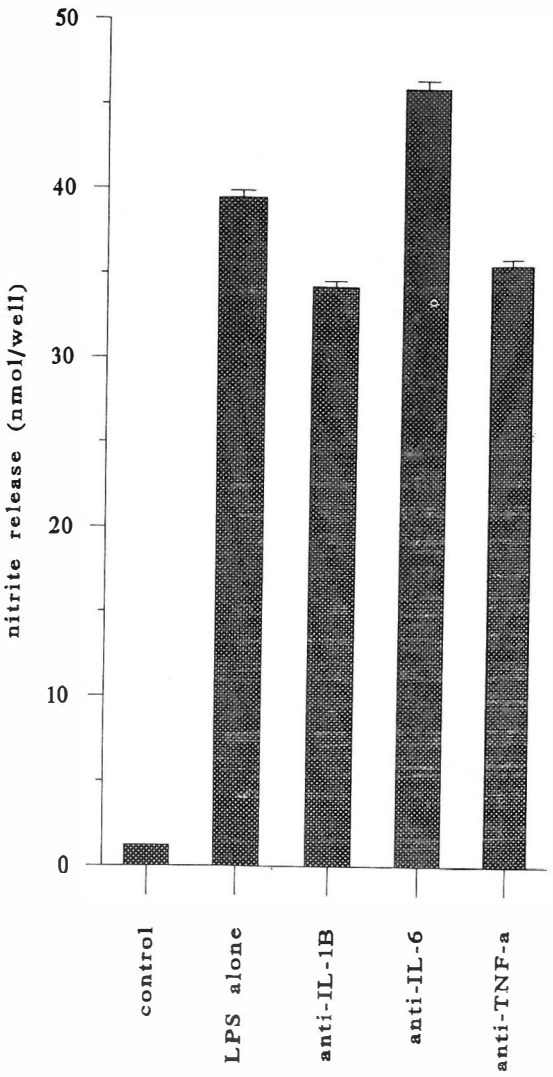

Fig. 2. Modulation of NO_2^- release by endogenous cytokines (n=5).

dependent increase in NO_2^- generation (Table I). 5α-DHT at 1×10^{-10} M enhanced NO_2^- production by 6.4% in males and 9.9% in females, at 1×10^{-9} M by 25% in males and 23.8% in females, and at 5×10^{-9} M by 39.3% in males and 24% in females. Moreover, maximum increase in NO_2^- release was induced in response to 1×10^{-8}M 5α-DHT [35.3% in males (p<0.005) and 29.5% in females (p<0.005)]. Incubation of activated peritoneal macrophages either from male or female mice with concentrations of 5α-DHT in excess of 1×10^{-8}M induced no further increase in NO_2^- production. In order to ascertain whether androgens can modulate NO production directly in the absence of LPS or γ-IFN, the effect of 5α-DHT alone or in combination with LPS and γ-IFN was compared. As illustrated in Fig.1, 5α-DHT could not significantly enhance NO_2^- production by inactivated macrophages in both sexes. Figure 1 shows that the level of NO_2^- production in inactivated macrophages from male mice is 2.4 times greater than in female mice (p<0.01).

Our results illustrated in Fig. 2 indicate that both IL-1β and TNF-α, endogenously released by macrophages in culture had a positive feedback on nitrite production. This is suggested by Ab neutralization of the cytokines which results in a slight decrease in NO_2^- production. On the other hand, blocking endogenous IL-6 with anti-IL-6 monoclonal Ab significantly (p<0.05) increased NO_2^- indicating that IL-6 might have a negative feedback on NO production by macrophages.[17]

DISCUSSION

After showing the effect of 5α-DHT on cytokine[5] and also nitric oxide[9] production by mice peritoneal macrophages,

the next step was to find out if 5α-DHT modulates NO release through cytokine production. The results obtained in this study showed that 5α-DHT enhanced LPS and γ-IFN-induced production of NO_2^- in a concentration-dependent manner. On the other hand, 5α-DHT was unable to affect NO release in inactivated Mϕ. Therefore, it seems that LPS upregulate the receptor for 5α-DHT. In this respect, we have previously demonstrated that androgen binding sites in peritoneal Mϕ have not been detected in the absence of LPS.[18] It has also been reported that 5α-DHT, at physiological concentrations, significantly ($p<0.05$) enhances NO release by peritoneal macrophages.[9]

Endogenous IL-1β and TNF-α are two potent stimulators of NO_2^- production, and neutralization of these two cytokines in tissue culture resulted in decreased NO_2^- production by LPS-stimulated macrophages (Fig. 2). These results are in agreement with a previous study by Liew,[19,20] who reported that exogenous cytokines like TNF-α, IL-1 and γ-IFN have a synergistic effect with LPS in inducing the production of the enzyme NO_3^- synthetase in a variety of cells and tissue. Moreover, neutralization of endogenous IL-6 by anti-IL-6 monoclonal Ab (Fig. 2) resulted in increased NO_2^- production by LPS-stimulated macrophages. On the other hand, Hatzigeorgiou[17] demonstrated that IL-6 down - modulated the anti-leishmanial activity of cytokine-activated macrophages. In addition, Bermudez[21] reported that exogenous IL-6 (10U/mL) abrogated TNF-α (10^3 U/mL) activation of macrophage killing of *M. avium* and *Leishmania* by both oxygen-dependent and independent mechanisms. However, reactive nitrogen intermediates have been implicated as a mechanism for the killing of intracellular parasites and bacteria such as *Leishmania, T. cruzi, M. tuberculosis, M. avium* and *T. gondii*.[17]

Therefore it seems that IL-6, by down-modulating NO production, decreases the anti-leishmania activity of cytokine-activated macrophages.

On the other hand, the male sex hormone testosterone is known to exacerbate a wide variety of disease processes and worsen the course and outcome of many infectious diseases caused by viruses, bacteria, protozoan and helminthic parasites.[22]

Taken together, these findings and the data presented by others demonstrate that the male hormone 5α-DHT may modulate NO release through cytokine production and part of the sex differences in immune response to infectious disease might be related to this modulation.

REFERENCES

1. Gueme PA, Carson DA, Lotz M: IL-6 production by human articular chondrocytes: modulation of its synthesis by cytokines, growth factor, and hormones *in vitro*. J Immunol 144 (2): 499-505, 1990.
2. Li ZG, Danis VA, Brooks PM: Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells *in vitro*. Clin Exp Rheumatol 11: 157-162, 1993.
3. Cutolo M, Accardo S, Balleari E, Sulli A, Seriolo B, Giusti M: Androgen status in postmenopausal patients with rheumatoid arthritis. J Rheum 20(12): 2163-65, 1993.
4. Satoskar A, Alexander J: Sex-determined susceptibility and differential IFN-γ and TNF-α mRNA expression in DBA/2 mice infected with *Leishmania mexicana*. Immunol 84: 1-4, 1995.
5. Ahmadi K , McCruden AB: Effect of 5α-DHT on cytokine production by peritoneal macrophages of NZB/BALBc mice. Med J Islam Rep Iran 11(3): 223-28, 1997.
6. Cannon J, Dinarelli CA: Increased plasma IL-1 activity in women after ovulation. Science 227: 1247-1249, 1985.
7. Flynn A: Stimulation of IL-1 production from placental monocytes. Lymphokine Research 3(1): 1-5, 1984.
8. Flynn A: Expression of Ia and the production of IL-1 by peritoneal exudate macrophages activated *in vivo* by steroids. Life Science 38: 2455-60, 1986.
9. Ahmadi K, McCruden AB: Sex differences in macrophage nitric oxide production. Iran J Med Sci 23 (1,2): 42-47,1998.
10. Liew FY, Li Y, Severn A, et al: A possible novel pathway of regulation by murine T helper type-2 (Th2) cells of a Th1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. Eur J Immunol 21: 2489-94,1991.
11. Leal LMCC, Moss DW, Kuhn R, Muller W, Liew FY: Interleukin-4 transgenic mice of resistance background are susceptible to *Leishmania major* infection. Eur J Immunol 23: 566-69,1993.
12. Cunha FQ, Moncada S, Liew FY: Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthetase by interferon-gamma in murine macrophages. BiochemBiophys Res Comm 182: 1155-59, 1992.
13. Ding AH, Nathan CF, Graycar J, Derynck R, Stuehr DJ, Srimal S: Macrophage deactivating factor and transforming growth factor-beta1, beta2 and beta3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. J Immunol 145: 940-44, 1990.
14. Cunha RQ, Weiser WY, David JR, Moss D V, Moncada S, Liew FY: Recombinant migration inhibitory factor induces nitric oxide synthase in murine macrophages. J Immunol 150: 1908-12, 1993.
15. Chao CC, Gekker G, Hu S, Peterson PK: Human microglial cell defence against *Toxoplasma gondii*; the role of cytokines. J Immunol 152: 1246-52, 1994.
16. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JJ, Tannenbaum SS: Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Analytical Biochemistry 126: 131-138, 1982.
17. Hatzigeorgiou E, He S, Sobel J, Grabstein K H, Hafner A, Ho JL: IL-6 down-modulates the cytokine-enhanced antileishmanial activity in human macrophages. J Immunol 151 (7): 3682-92, 1993.
18. Ahmadi K, McCruden AB: Androgen binding site in macrophages. Iranian J Med Sci 22 (1, 2): 32-37, 1997,
19. Liew F Y: The role of nitric oxide in parasitic diseases. Annal Trop Med Parasitol 87 (6): 637-42, 1993.
20. Liew F Y: Regulation of nitric oxide synthesis in infectious and autoimmune disease. Immunol Letters 43: 95-98, 1994.

21. Bermudez LE, Wu M, Petrofsky M, Young LS: IL-6 antagonizes tumor necrosis factor-mediated mycobacteriostatic and mycobactericidal activities in macrophages. Infect Immun 60: 4245-52, 1992.

22. Benten WPM, Wunderlich F Herrmann R, Kuhn-Velten WN: Testosterone-induced compared with oestradiol-induced immunosuppression against *Plasmodium chabaudi* malaria. J Endocrinol 139: 487-94, 1993.