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

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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.

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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.1-3 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.4-9

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
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<table>
<thead>
<tr>
<th>No.</th>
<th>5α-DHT/M</th>
<th>Male (NO₂/nM/well)</th>
<th>Female (NO₂/nM/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>45.2</td>
<td>49.4</td>
</tr>
<tr>
<td>2</td>
<td>1×10⁻¹³</td>
<td>44.9</td>
<td>50.3</td>
</tr>
<tr>
<td>3</td>
<td>1×10⁻¹²</td>
<td>45</td>
<td>50.9</td>
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<tr>
<td>4</td>
<td>1×10⁻¹¹</td>
<td>45.2</td>
<td>51.5</td>
</tr>
<tr>
<td>5</td>
<td>1×10⁻¹⁰</td>
<td>48.1</td>
<td>54.3</td>
</tr>
<tr>
<td>6</td>
<td>1×10⁻⁹</td>
<td>56.5</td>
<td>61.2</td>
</tr>
<tr>
<td>7</td>
<td>5×10⁻⁹</td>
<td>63</td>
<td>61.3</td>
</tr>
<tr>
<td>8</td>
<td>1×10⁻⁸</td>
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<tr>
<td>10</td>
<td>1×10⁻⁶</td>
<td>63.3</td>
<td>64.2</td>
</tr>
</tbody>
</table>

inhibit NO expression. 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.

**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. 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 1×10⁻⁶M to 1×10⁻¹³M were added and cells incubated at 37°C at 5% CO₂ 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 further 24h. 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. 50 or 100 μL aliquots were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylendiamine dihydrochloride (Sigma) and 2.5% H₃PO₄) 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. NO₂ was determined using sodium nitrite (NaN₃) as a standard. The concentration of NO₂ obtained in the experiments was corrected by subtracting of NO₃ 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 (1×10⁻⁶M to 1×10⁻¹³M) for 24 h induced a concentration-
dependent increase in NO\textsubscript{2} generation (Table I). 5α-DHT at 1 \times 10^{-10} M enhanced NO\textsubscript{2} production by 6.4% in males and 9.9% in females, at 1 \times 10^{-9} M by 25% in males and 23.8% in females, and at 5 \times 10^{-9} M by 39.3% in males and 24% in females. Moreover, maximum increase in NO\textsubscript{2} release was induced in response to 1 \times 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 \times 10^{-8} M induced no further increase in NO\textsubscript{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\textsubscript{2} production by inactivated macrophages in both sexes. Figure 1 shows that the level of NO\textsubscript{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\textsubscript{2} production. On the other hand, blocking endogenous IL-6 with anti-IL-6 monoclonal Ab significantly \((p<0.05)\) increased NO\textsubscript{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\textsuperscript{2} and also nitric oxide\textsuperscript{6} production by mice peritoneal macrophages,
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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 NO₂⁻ in a concentration-dependent manner. On the other hand, 5α-DHT was unable to affect NO release in inactivated M₀. Therefore, it seems that LPS upregulate the receptor for 5α-DHT. In this respect, we have previously demonstrated that androgen binding sites in peritoneal M₀ have not been detected in the absence of LPS. It has also been reported that 5α-DHT, at physiological concentrations, significantly (p<0.05) enhances NO release by peritoneal macrophages.

Endogenous IL-1β and TNF-α are two potent stimulators of NO₂⁻ production, and neutralization of these two cytokines in tissue culture resulted in decreased NO₂⁻ production by LPS-stimulated macrophages (Fig. 2). These results are in agreement with a previous study by Liew, 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 NO₃ 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 NO₂⁻ production by LPS-stimulated macrophages. On the other hand, Hatzigeorgiou demonstrated that IL-6 down-modulated the anti-leishmanial activity of cytokine-activated macrophages. In addition, Bermudez reported that exogenous IL-6 (100 U/mL) abrogated TNF-α (10 ng/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.

Therefore it seems that IL-6, by down-modulating NO production, decreases the anti-leishmanial 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.

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

