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STIMULATION OF α-ADRENERGIC RECEPTOR AUGMENTS THE PRODUCTION OF MACROPHAGE-DERIVED TUMOR NECROSIS FACTOR[1]

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Accumulating evidence supports the hypothesis that neuroendocrine hormones may participate in immunologic processes. In our study we have determined that UK-14304 (UK) and norepinephrine (NE), both α_2-adrenergic agonists, can augment LPS-stimulated TNF from elicited macrophages (MO). The increase in TNF production was concentration dependent with an EC_{50} for UK and NE of 8.1 ± 2.6 and 0.52 ± 0.17 nM, respectively. The concentration-effect curve for UK and NE was shifted to the right by the α_2-antagonist yohimbine (10^{-6} M), with new EC_{50} of 49.7 ± 12.2 ($p < 0.001$) nM and 10.3 ± 22 nM. The augmenting effect of UK on MO TNF production was assessed over a 7 log LPS response curve. Within a single population of MO 10 nM UK shifted the LPS-induced TNF curve eightfold to the left with the greatest increase in TNF production at lower LPS concentrations. At the transcriptional level, Northern blot analysis demonstrated that UK increased LPS-induced TNF mRNA accumulation. This augmentation in TNF mRNA accumulation was blocked by yohimbine. The presence of a MO α-adrenergic receptor was established by demonstrating binding of the α_2-adrenergic antagonist ^{3}H-yohimbine to membranes prepared from MO. This binding was rapid, saturable, reversible, and blocked by UK, clonidine, and phentolamine. These investigations support the role of α_2 adrenergic agonists as immunostaining compounds that may regulate cytokine production during an inflammatory response.

MO[3] are recognized as important immune cells involved in the initiation and maintenance of an inflammatory response via the production of specific cytokines. These MO-derived polypeptide mediators, such as TNF, serve as important cell-to-cell communication signals needed to orchestrate an effective host defense; yet, knowledge regarding processes that regulate TNF is limited. Inasmuch as MO-derived TNF may be necessary for the proper maintenance of inflammation, it is important to understand endogenous mechanisms that control the production of this cytokine.

One class of endogenous mediators that may potentially dictate MO responsiveness are neuroendocrine hormones. These signals derived from the central nervous system appear to be integrated into the immune response and participate in neuro-immune communication. Recent studies have demonstrated that this communication network is actively engaged during an inflammatory response and stress (1–3). In particular, adrenergic agents have been shown to have a immunoregulating effect on MO responsiveness (4–9). Agents that act via β-adrenergic receptors have shown a reduction in immune cell activity, but only at micromolar concentrations. On the contrary, stimulation of cells via α-adrenergic receptors have demonstrated an augmentation in immune cell responsiveness. The catecholamine norepinephrine can augment MO phagocytosis and tumoricidal activity at concentrations that are consistent with those previously reported for stimulating the α-adrenergic receptor (10). Our data demonstrating that MO possess a functional α_2-adrenergic receptor that can bind the antagonist yohimbine in a rapid, saturable, and reversible manner. Scatchard analysis demonstrated that this receptor has a binding site of high affinity with a K_d of 6.4 nM. Stimulation of the MO α_2-adrenergic receptor with either norepinephrine or UK-14304 significantly augmented LPS-induced MO TNF production, with an apparent affinity similar to that obtained in binding assays, thus demonstrating functional activity associated with receptor activation. These studies support the concept of communication networks linking neurologic and immunologic systems.

MATERIALS AND METHODS

Murine MO cultures. Female, CBA/J, specific pathogen-free mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Mice were housed in pathogen-free quarters and given food and water ad libitum. Elicited murine MO were recruited via a single intraperitoneal injection of 0.5 ml CFA (diluted 1/1 with sterile saline). After 14-day the activated peritoneal MO (more than 80% Ia$^+$) were harvested, washed extensively, and resuspended in sterile RPMI 1640 (Whitaker Biomedical Products, Whitaker, CA), 1 mM glutamine, 25 mM HEPES, 100 U penicillin, and 100 μg streptomycin/mL. MO (1×10^6/ml) were added to either 35 mm (1 ml) or 100 mm (10 ml) culture dishes and incubated at 37°C in 5% CO_2/95% humidified air. After 2 h adherence, the cells were washed three times and the MO monolayers were overlaid with media containing the appropriate agents described below. The use of peritoneal MO precluded the role of platelets in this cell system.

Membrane preparation. Adherent, monolayer MO (approximately

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[3] Abbreviations used in this paper: MO, macrophage; UK, UK-14304; NE, norepinephrine.

3×10^8 cells) were scraped in ice-cold sterile RPMI 1640 and collected into a single pellet by centrifugation at 1000 × *g* for 10 min. Cell pellets were homogenized with 20 strokes of a motor-driven Teflon-tipped pestle at moderate speed in 6 ml of ice-cold Tris-sucrose buffer (Tris-HCl, 5 mM; sucrose, 250 mM; $MgCl_2$, 1 mM; pH 7.4). The homogenates were centrifuged at 40,000 × *g* for 10 min, and the pellet was washed twice in fresh ice-cold incubation buffer (Tris-HCl, 40 mM; $MgCl_2$, 10 mM; pH 7.5) and recentrifuged at 40,000 × *g* for 10 min. The final pellet was resuspended in 20 ml of incubation buffer, resulting in a protein concentration of about 0.1 to 0.3 mg/ml. Protein concentrations were determined by using a standard protein assay (BCA, Pierce Chemical Company, Rockford, IL).

[^{3}H] yohimbine binding assay. The procedure for radioligand binding studies was a modification of a procedure for specific binding of radioligands to neuronal tissue (11). For the equilibrium studies total [^{3}H]yohimbine binding was measured in 500-μl aliquots of fresh cell suspension in the incubation buffer. Samples were incubated in duplicate for 30 min at 22°C with 0.25 to 32 nM [^{3}H] yohimbine (sp. act. 72.5 Ci/mmol, NEN, Boston, MA) in a Dubnoff shaking incubator. Nonspecific binding was determined by adding unlabeled yohimbine hydrochloride, 10 μM, (Sigma Chemical Co., St. Louis, MO) to one of the duplicate samples. Specificity of [^{3}H]yohimbine was defined as the binding in excess of nonspecific interactions. Under these assay conditions, specific binding of 2 nM [^{3}H]yohimbine was 70 to 80%. In drug competition and kinetic studies the fresh membranes were incubated with 8×10^{-9} M [^{3}H]yohimbine.

Incubations were terminated by diluting the sample with 3.5 ml of Tris incubation buffer (22°C), followed by rapid filtration under vacuum through GF/C glass fiber filters. The filters were immediately washed with two 10-ml aliquots of Tris incubation buffer (22°C) and air dried. Apparent dissociation constants and the maximum number of binding sites (B_{max}) for [^{3}H]yohimbine were determined using Scatchard and Hill transformations analysis (12). For drug competition studies the percentage of inhibition of [^{3}H]yohimbine binding obtained at various drug concentrations was fitted using the nonlinear least squares curve fitting program, GRAPH PAD (Institute for Scientific Information, Graph Pad Software, San Diego, CA). EC_{50} is the concentration of drug that produces half maximal inhibition.

Experimental protocol. Initial experiments were designed to assess the effect of UK (Pfizer Co., Sandwich, Kent) on MO TNF production in response to graded concentrations of LPS (*Escherichia coli* 0111:B4, Sigma). MO in monolayer were incubated in the presence or absence of UK (10^{-8} M) plus graded concentrations of LPS (1 pg/ml to 1 μg/ml). After 4 h incubation, cell-free supernatants were collected and stored at −20°C until bioassayed. In subsequent studies, MO were challenged with various concentrations of UK or NE in the presence or absence of yohimbine (10^{-6} M) plus LPS (10 μg/ml). In the latter experiments, the sensitivity of the agonists were assessed. Four hours post-LPS addition, the cell-free supernatant was recovered for TNF bioassay and the adherent MO were processed for TNF mRNA analysis. The percent potentiation was determined using the following formula:

$$\frac{\text{(LPS + UK or other } \alpha_2\text{-agonists)} - \text{(LPS alone)}}{\text{LPS alone}}$$

TNF analysis. Cell-free supernatants from treated MO monolayers were assessed for bioactivity using the LM fibroblast cytolytic assay (13). LM cells (5×10^4/0.1 ml) were cultured in 96-well microtiter plates with serial dilutions (1/2) of test samples and 1 μg/well actinomycin D. TNF-α standard consisted of rTNF (22 U/ng; Cetus Corp., Emeryville, CA), which was serially diluted. After 18 h incubation at 37°C the plates were washed and the remaining viable cells were stained with crystal violet (0.5% in methanol/water 1:4). The level of cell lysis was determined using a MicroELISA Autoreader (620 nm) (Bio-Tek, Winooski, VT). Units of activity were derived from the TNF standard curve. The sensitivity of the assay was more than 5 U/ml. Neither UK, NE, nor yohimbine (10^{-8} to 10^{-5} M) had a direct influence on the LM bioassay.

MO TNF mRNA analysis. Total MO RNA was isolated according to a modification of the procedures established by Chirgwin et al. (14) and Jonas et al. (15). The MO monolayer from a 100-mm plate (20×10^6 total MO) was solubilized in a solution of 25 mM Tris, pH 8.0, containing 4.2 M guanidium isothiocyanate, 0.1 M 2-ME, and 0.5% sarkosyl. After homogenization, an equal volume of 100 mM Tris, pH 8.0 containing 10 mM EDTA and 1.0% SDS was added and the entire solution was extracted with chloroform-phenol and chloroform-isoamyl alcohol. The extracted RNA was precipitated in alcohol and the pellet dissolved in 10 mM Tris/0.1 mM EDTA buffer with 0.1% sarkosyl. Separation of RNA was performed using a formaldehyde/1% agarose gel. The RNA was transblotted to nitrocellulose and TNF mRNA was probed using a ^{32}P-labeled oligonucleotide(5′-GTC-CCC-CTT-CTC-CAG-CTG-GAA-GAC-TCC-TCC-3′)(14). The filters were hybridized for 16 h, washed in 6× SSC and exposed with an intensifying screen to X-omat R film (Eastman Kodak, Rochester, NY) at −70°C. The autoradiographs were quantitated by laser densitometer scan. The total RNA levels per lane on the gel was assessed by monitoring 28S and 18S rRNA and expression of actin mRNA. Accumulation of β-actin mRNA was determined by probing the same blot with a ^{32}P-labeled 42-mer oligonucleotide (5-GGC-TGG-GGT-GTT-GAA-GGT-CTC-AAA-CAT-GAT-CTG-GGT-CAT-CTT-3′) (15).

Statistics. Complete concentration-effect relationships for LPS, UK, and NE were determined to establish changes in the TNF response. The percentage of response obtained at various LPS, UK, or NE concentrations was obtained by using nonlinear least squares regression to fit a curve. All data are expressed as means values ± the SEM. Statistical significance was evaluated with a paired Student's *t* test.

RESULTS

Effect of UK on concentration-effect curve of LPS. The production of TNF by LPS-stimulated MO was found to be dose dependent, as LPS concentrations between 10 pg and 100 ng induced a near linear increase in TNF levels. Low TNF levels were observed with as little as 1 pg/ml LPS, although maximum response occurs when MO were treated with 50 ng/ml (Fig. 1). The specific α_2-adrenergic receptor agonist, UK-14304, demonstrated an augmenting effect on LPS-induced TNF production. The LPS concentration-effect curve was significantly shifted to the left from an EC_{50} of 6.05 ± 2.5 ng/ml for LPS alone to 0.79 ± 0.2 ng/ml ($p < 0.05$) for LPS in the presence of UK (10^{-8} M).

Concentration-dependent effect of NE and UK on release of TNF during LPS stimulation. The effect of different concentrations of NE and UK were evaluated to determine whether both α_2-agonists could potentiate LPS-stimulated TNF release in a concentration-dependent manner (Fig. 2 *A* and *B*). Graded concentrations of either NE or UK added simultaneously with LPS (30 ng/ml) potentiated TNF release over LPS alone. The EC_{50} for the NE concentration-effect curve was 0.52 ± 0.17 nM. The addition of the α_2-antagonist yohimbine (10^{-6} M), significantly shifted the NE concentration-effect curve to the right with an EC_{50} of 10.32 ± 2.19 nM ($p < 0.001$). In a similar manner, UK potentiated LPS-stimulated TNF production with an EC_{50} of 8.1 ± 2.6 nM. As with the NE experiment, the UK concentration-effect curve was significantly shifted to the right by yohimbine (10^{-6} M) to an EC_{50} of 49.7 ± 12.2 nM ($p < 0.001$). In these studies, UK did not appear to prime macrophages for an augmented response to LPS, as the addition of an α_2 adre-

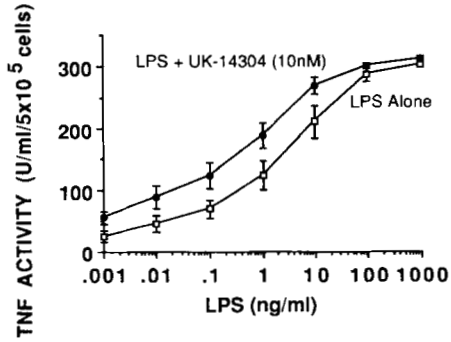

Figure 1. Effect of UK on the concentration-effect curve of LPS. MO were challenged with LPS (1 pg/ml to 1 μg/ml) alone or in the presence of UK (10^{-8} M). Supernatants were assayed for TNF 4 h later. Each point represents the mean of 4 to 11 determinations ± SEM per each data point.

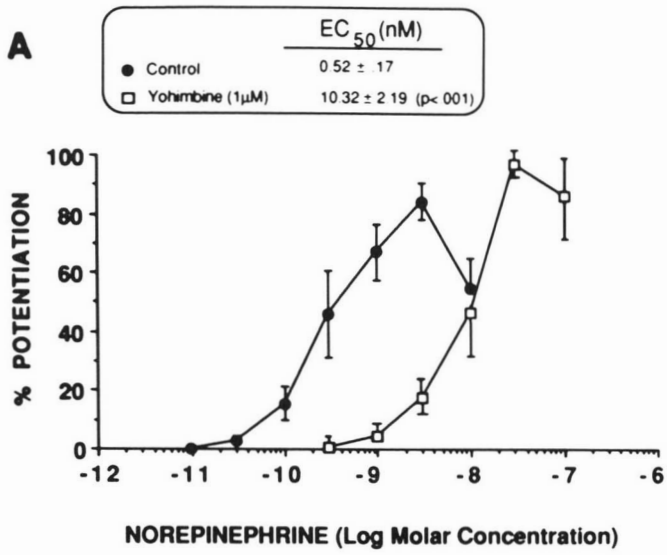

Figure 2. *A*, Potentiation of the release of TNF by NE during LPS stimulation of MO and the effect of yohimbine on the NE response. Potentiation of TNF release is expressed as a percent of the total amount of TNF released from MO in the presence of LPS alone. MO were challenged with LPS (30 ng/ml) alone and with different concentrations of NE ± yohimbine (10^{-6} M). In the above data 30 ng/ml of LPS alone resulted in the production of 260 U of TNF, whereas UK plus LPS maximally generated 515 U LPS; *B*, Potentiation of the release of TNF by UK during LPS stimulation of MO and the effect of yohimbine on the UK response. MO were challenged with LPS (30 ng/ml) alone and with different concentrations of UK ± yohimbine (10^{-6} M); supernatants were assayed for TNF 4 h later. Each point represents six to nine determinations ± SEM per each data point.

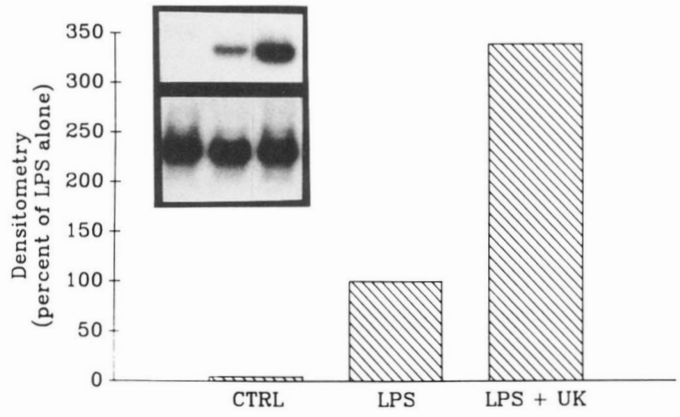

Figure 3. Effects of UK (10^{-8} M) on LPS-induced TNF mRNA accumulation. MO were challenged with LPS (30 ng/ml) alone or in the presence of UK (10^{-8} M) for 3 h and northern analysis was performed on RNA extracts taken 3 h after LPS challenge. The *upper blot* is probed for TNF-α and the *lower blot* is probed for actin.

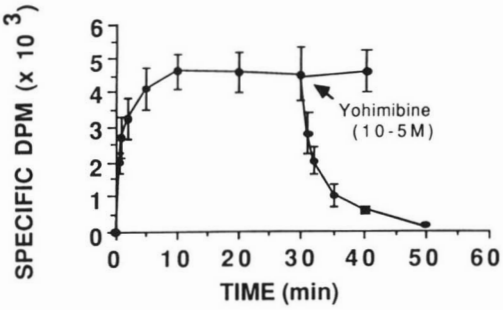

Figure 4. Kinetics of specific binding of [^{3}H]yohimbine (8 nM) to MO. MO membranes were preincubated for 10 min at 22°C followed by incubation in duplicate with 8 nM [^{3}H]yohimbine in the absence (total binding) or presence (nonspecific binding) of cold 10^{-5} M yohimbine. For the reversal of binding, MO membranes were incubated at 22°C for 30 min with [^{3}H]yohimbine (8 nM), followed by addition of cold yohimbine (10^{-5} M). At varying times, both the total and nonspecific tubes were removed and the reaction was stopped and the samples were filtered and counted as described in *Materials and Methods*. Each point is the mean value ± SEM of three experiments performed in duplicate.

nergic agonist before LPS did not change the LPS responsiveness. Thus, the augmenting effects of the α_2-agonists UK and NE upon LPS-induced TNF production was blocked by the α_2-receptor antagonist yohimbine.

Effect of UK on TNF mRNA isolated from LPS challenged MO. The simultaneous administration of UK with LPS demonstrated an increase in TNF mRNA accumulation over LPS alone (Fig. 3). The UK potentiation of LPS-induced TNF mRNA is LPS dependent, because the administration of UK without LPS did not induce the expression of TNF mRNA (data not shown). The simultaneous addition of UK and LPS resulted in over a threefold increase in steady state TNF mRNA accumulation, as assessed by Northern blot analysis. In these studies, the Northern blots were quantitated by laser densitometry (Fig. 3).

Kinetics and specificity of [^{3}H]yohimbine binding to MO membranes. The specific binding of [^{3}H]yohimbine was rapid, saturable and reversible using fresh MO membranes (Fig. 4). At 22°C the association reaction reached equilibrium within 10 min. In the presence of 10^{-5} M unlabeled yohimbine the bound [^{3}H]yohimbine was rapidly dissociated. In equilibrium saturation experiments, nonspecific binding increased in a linear manner with increasing [^{3}H]yohimbine concentrations (data not shown). Scatchard analysis demonstrated a curvilinear plot (Fig. 5) with two binding components possessing K_d values for [^{3}H]yohimbine of 6.4 ± 1 and 112.6 ± 31 nM. The high-affinity class had a capacity of 231 ± 28 fmol/mg protein and the low affinity class of sites has a capacity of 1560 ± 243 fmol/mg protein. Hill analyses of both affinity sites for [^{3}H]yohimbine binding indicated that there was no cooperativity (n_H not different from 1.0).

To further characterize the alpha-adrenergic receptor on mouse MO we compared K_i values for the inhibition of [^{3}H]yohimbine by selective adrenergic drugs (Table I). Yohimbine, clonidine, and UK (agents that selectively bind the α_2-adrenergic receptor) were potent in displacing [^{3}H]yohimbine binding. Furthermore, phentolamine had

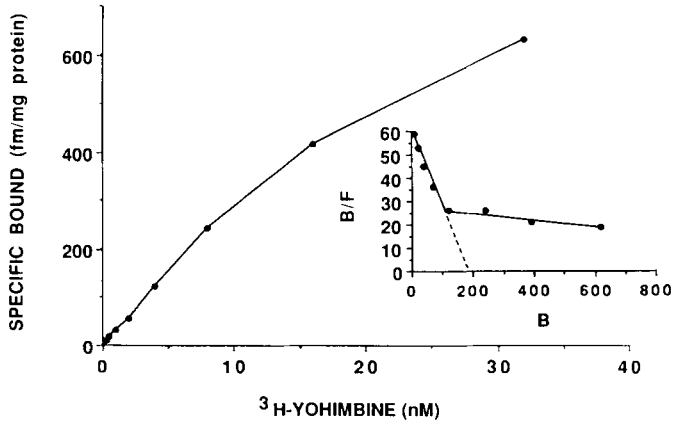

Figure 5. Binding isotherms and Scatchard analysis of [^{3}H]yohimbine binding to MO membranes as a function of increasing concentrations of the radioligand. Membranes were incubated with the radioligand in the absence (total binding) or presence (nonspecific binding) of cold 10^{-5} M yohimbine. Representative Scatchard plot showing the existence of two binding sites for [^{3}H]yohimbine, one of high affinity (K_d = 6.4 nM; B_{max} = 231 fmol/mg protein) and a second site of lower affinity and higher capacity (K_d = 112 nM; B_{max} = 1560 fmol/mg protein). The data shown are representative of those obtained in nine experiments.

TABLE I

Inhibition of [^{3}H]yohimbine binding at 8 nM[a]

Drug	High-Affinity Site K_i (nM)	Low-Affinity Site K_i (nM)	n
Yohimbine	0.83 ± 0.24	1229 ± 224	8
UK-14304	0.80 ± 0.08	357 ± 78	5
Clonidine	1.1 ± 0.89	3969 ± 680	6
Phentolamine	427 ± 148		6
Propranolol	>10,000		3

[a] Inhibition of specific [^{3}H]yohimbine binding to MO membranes by various adrenergic drugs. Membranes were incubated for 20 min at 22°C with 8 nM [^{3}H]yohimbine and various concentrations of competing agents. K_i values were calculated by the equation from Cheng and Prussoff (27): $IC_{50}/1 + ((\text{radioligand})/K_d)$. Each value is the mean ± SEM of n determinations.

400 times less potency than the above compounds suggesting the binding site is not identical to other characterized α_2-adrenergic receptors. The rank order of potency for selective adrenergic drugs (UK > yohimbine > clonidine > phentolamine > propranalol) was consistent for an α_2-adrenergic-like receptor site.

DISCUSSION

Studies from a number of laboratories have established that functional communication links exist between the neurologic and immunologic systems (7, 10, 16–19). This communication network appears to be maintained by molecular signals that are both tonically produced and secreted upon stimulation. Although a number of these mediators may be unique to either the immune or neurologic system, others appear to possess overlapping activities. For example, adrenergic compounds have been shown to influence leukocyte function (9, 10), although substance P and substance K have been demonstrated to augment macrophage-derived IL-1 and TNF-α (16, 17). This latter activity may be especially important to the altered physiology found in populations of stressed individuals (20), as TNF can induce both weight loss and hypothermia. In addition, neuroimmunomodulation may account for stress induced changes leading to an altered inflammatory response (3, 21).

These data further demonstrate a neuroimmune link, as the stress-associated catecholamine norepinephrine can regulate MO-derived TNF via α-adrenergic receptor interactions. Both norepinephrine and the α_2-adrenergic agonist, UK-14304 was shown to augment LPS-induced TNF production. This augmentation was prevented by the α_2-adrenergic antagonist yohimbine. In addition, Northern blot analysis demonstrated that the augmenting effect of UK-14304 may be a result of an increase in steady state TNF mRNA. The presence of an α_2-adrenergic-like receptor on MO membranes was determined by direct binding of [^{3}H]yohimbine, as well as cold competition studies. These binding data are consistent with previous reports that demonstrated a putative α_2-adrenergic receptor on MO membranes (22), as well as an α_2-adrenergic receptor with both high and low affinity sites for yohimbine on neutrophils (23). In the former study, L-epinephrine-treated MO resulted in a suppression of PGE_2-induced adenylate cyclase activity (22). The activity of an additional immune cell, the lymphocyte, has also been shown to be augmented by the α_2-receptor agonist UK-14304 (24).

Although a number of studies have demonstrated that activation of the MO β-adrenergic receptor can result in a suppressive effect (8, 25, 26), little is known regarding events that occur upon activation of the MO α_2-adrenergic receptor. Although α-adrenergic responses have been suggested to occur on MO (7, 8, 22) no physiologic or pharmacologic role has been well established. Our results demonstrate that MO membranes contain both high and low affinity binding sites for α_2-adrenergic receptor ligands. Furthermore, our study demonstrates that activation of the α_2-adrenergic receptor on MO results in an augmentation of LPS-induced TNF. Macrophages treated with LPS in the presence of an α_2-adrenergic receptor agonist result in a dose-dependent potentiation in the production of TNF that was blocked by yohimbine. Northern blot analysis demonstrated that steady state TNF mRNA was augmented in LPS + UK-14304-treated cells. These data support the concept that communication circuits between MO and the neurologic systems are dynamic processes which may dictate events induced during homeostasis, stress, and disease.

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