Activation of the Adenosine A₃ Receptor in RAW 264.7 Cells Inhibits Lipopolysaccharide-Stimulated Tumor Necrosis Factor-α Release by Reducing Calcium-Dependent Activation of Nuclear Factor-κB and Extracellular Signal-Regulated Kinase 1/2

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ABSTRACT

Bacterial lipopolysaccharide (LPS) activates the immune system and promotes inflammation via Toll-like receptor (TLR) 4, which regulates the synthesis and release of tumor necrosis factor (TNF)-α and other inflammatory cytokines. Previous studies have shown that the nucleoside adenosine suppresses LPS-stimulated TNF-α release in human U937 macrophages by activating an adenosine A₃ receptor (A₃AR) subtype on these cells. In this study, we examined the mechanism(s) underlying A₃AR-dependent inhibition of TNF-α release in a mouse (RAW 264.7) cell line. Treatment of RAW 264.7 cells with LPS (3 μg/ml) increased TNF-α release, which was reduced in a dose-dependent manner by adenosine analogs N⁶-(3-iodo-benzyl)-adenosine-5'-N-methyluronamide (IB-MECA) and R-phenylisopropyladenosine and reversed by selective A₃AR blockade. The increase in TNF-α release was preceded by an increase in intracellular Ca²⁺ levels. Inhibition of intracellular Ca²⁺ release by IB-MECA, a selective agonist of the A₃AR, or with BAPTA-AM, an intracellular Ca²⁺ chelator, reduced LPS-stimulated TNF-α release. Activation of the A₃AR or inhibition of intracellular Ca²⁺ release also reduced LPS-stimulated nuclear factor-κB (NF-κB) activation and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Similar inhibition by A₃AR was observed for LPS-stimulated inducible nitric-oxide synthase. These data support the contention that inhibition of LPS-stimulated release of inflammatory molecules, such as TNF-α and NO via the A₃AR, involves suppression of intracellular Ca²⁺ signaling, leading to suppression of NF-κB and ERK1/2 pathways.

Gram-negative bacteria represent a major group of pathogens responsible for causing serious infections. The lethal effect of such infections is linked to the biological effects of a glycolipid, lipopolysaccharide (LPS), or endotoxin (Morrison and Ryan, 1987). LPS activates cell signaling through the Toll-like receptor (TLR) 4 (Janeway and Medzhitov, 2002). All TLR activate a common signaling pathway, which culminates in the activation of nuclear factor-κB (NF-κB) transcription factors, as well as the mitogen-activated protein kinases (MAPK) (Adem and Ulevitch, 2000). This process leads to the transcriptional activation of genes involved in the inflammatory response, such as inducible nitric-oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and interleukin (IL)-1 (Barnes and Karin, 1997).

In the unstimulated cells, NF-κB is localized to the cytoplasm, where stimulus-induced activation promotes its translocation to the nucleus to transactivate NF-κB target genes. The translocation process is initiated by stimulus-mediated phosphorylation and degradation of IκB, an inhibitory binding protein present in the cytosol, releasing the

ABBREVIATIONS: LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; AR, adenosine receptor; IB-MECA, N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; R-PIA, R-phenylisopropyladenosine; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase(s); PDTCC, pyrrolidine dithiocarbamate; PD98059, 2'-amino-3'-methoxyflavone; MRS1220, N-(9-chloro-2-2-furyl)1,2,4-triazolo[1,5-c]quinazolin-5-benzeneacetamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ZM241385, 4-(2-[7-amino-2-(2-furyl)]1,2,4-triazolo[2,3-a][1,3,5]triazin-5-ylmethyl)phenol; iNOS, inducible nitric-oxide synthase; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid(tetraacetoxy-methyl)ester; EMSA, electrophoretic mobility shift assay(s); TLR, Toll-like receptor(s); IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NECA, 5'-N-ethylcarboxamidoadenosine.
active NF-κB homodimer or heterodimers, which is free to enter the nucleus and regulate gene expression (Karlin and Ben-Neriah, 2000).

MAPK are a group of related serine/threonine kinase nases, which also participate in LPS-mediated immune cell activation (Han et al., 1994; Dong et al., 2002). The MAPK family includes the extracellular signal-related kinase (ERK1/2, p38 MAPK, and c-Jun NH2-terminal kinase. These MAPK modulate cellular responses through phosphorylation of transcription factors and activation of downstream kinases. Inhibition of individual MAPK pathways suppresses downstream cytokine production. For example, inhibition of p38 MAPK reduces TNF-α and IL-8 (Foey et al., 1998; Manthey et al., 1998) and IL-10 levels (Foey et al., 1998) in LPS-stimulated monocytes. In contrast, inhibition of ERK produced only partial inhibition of TNF-α and IL-10 but no change in IL-1β production (Foey et al., 1998).

The nucleoside adenosine is a potent endogenous regulator of the inflammatory response by interacting with the P1 purinergic receptors. These receptors include the A1 adenosine receptor (AR), the A2A and A2B AR and the A3 AR subtypes (Olah and Stiles, 2000). Three of these, the A1, A2A and A3AR subtypes, are expressed on monocytes and macrophage cell lines (Sajjadi et al., 1996). Adenosine suppresses LPS-stimulated TNF-α production by macrophage lineage cells (Bouma et al., 1994; Hashko et al., 1996; Sajjadi et al., 1996), presumably through activation of the A2A (Bouma et al., 1994; Hashko et al., 1996) or the A3AR subtype (Hashko et al., 1996; Sajjadi et al., 1996). However, the mechanism mediating this anti-inflammatory action is not known.

This study was undertaken to better understand the mechanism by which adenosine mediates its anti-inflammatory action in a murine macrophage (RAW 264.7) line. This cell line is a widely used model for studying the effect of LPS on signal transduction pathways. Our data indicate that activation of the A3AR suppresses LPS-stimulated intracellular Ca2+ accumulation, resulting from inhibition of intracellular Ca2+ release and plasma membrane Ca2+ entry. We also show that a rise in intracellular Ca2+ promotes NF-κB and ERK1/2 activation, signaling pathways linked to TNF-α and iNOS production.

Materials and Methods

Materials. LPS (Escherichia coli 0111:B4), R-phenylisopropyladenosine (R-PIA), IB-MECA, EGTA, pyrrolidine dithiocarbamate (PDTC), and PD98059 were from Sigma-Aldrich (St. Louis, MO). MRS1220, DPCPX, and ZM241385 were from Tocris Cookson Inc. (Ellisville, MO). Antibodies for pERK1/2 and IκB-α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies for p42 ERK were from Calbiochem (San Diego, CA). ECL kits for Western blotting were purchased from ECL Western Blotting System (GE Healthcare, Little Chalfont, Buckinghamshire, UK). BAPTA-AM was from Calbiochem.

Cell Culture. The mouse macrophage cell line RAW 264.7 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5% CO2, and 100% humidity. Cells at 3 × 106/ml were added to culture plates, allowed to adhere overnight, and then stimulated with 3 μg/ml LPS. Adenosine receptor agonists, antagonists, and other drugs were added at the times indicated.

Radioligand Binding and Competition Assays. Cells were detached in ice-cold phosphate-buffered saline containing 5 mM EDTA. The cells were then lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 2 μg/ml pepstatin and homogenized briefly by Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Membranes were obtained by centrifugation of the homogenates at 40,000g for 15 min and were used for radioligand binding studies.

Radioligand binding was performed using the agonist radioligand [3H]IB-MECA. Membrane preparations (approximately 80 μg of protein) were incubated with the radioligands in the absence or presence of 1 mM theophylline. The reaction mixture was then filtered over GF/B glass fiber filters (Whatman, Clifton, NJ). The radioactive content of each filter was determined using a Packard (5780) gamma counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

Ca2+ Imaging. Cells were grown on coverslips, washed with physiological buffer (130 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, with pH adjusted to 7.3), and loaded with the Ca2+ indicator Fluo-4 AM (Invitrogen, Carlsbad, CA) at a concentration of 5 μM for 30 min at room temperature. Cells were then washed with physiological buffer (above), treated with the appropriate drugs, and imaged by confocal microscopy using an Argon laser at 488 nm. Images were recorded at baseline and scanned at 10-s intervals over a period of 6 min. Further analyses were performed using a Flouview software associated with the instrument. Data were collected as the average fluorescence detected from 20 cells per microscope field at each time point.

Measurement of TNF-α in Culture Supernatants. Cells were cultured with the various agents (as indicated) and incubated for 6 h. Supernatants were collected by removing the cell suspensions from the wells and centrifuging at 1500g for 5 min. The cell-free supernatants were transferred to clean tubes and stored at −20°C until TNF-α assays were performed.

Cytokine production was quantified using a sandwich ELISA technique. Supernatant levels of TNF-α were measured using OptEIA Mouse TNF-α (Mono/Mono) kits purchased from BD Biosciences Pharmingen (San Diego, CA). Assays were performed according to the manufacturer’s instructions. A standard curve was constructed using the recombinant TNF-α standard, and the concentration of the unknown was determined by linear regression analysis.

SDS-PAGE/Western Blotting. The cells were lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml pepstatin and homogenized briefly by Polytron homogenizer. The homogenates were centrifuged 40,000g for 15 min. For quantitation of ERK1/2, cells were incubated with phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich) prior to homogenization and supernatants were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, blocked in a solution containing 130 mM NaCl, 2.7 mM KCl, 1.8 mM Na2HPO4, 1.5 mM KH2PO4, 0.1% NaN3, 0.1% Triton X-100, and 5% low-fat skim milk for 2 h, and then incubated at 4°C overnight with the primary polyclonal antibody for IκB-α, phospho-ERK1/2, total ERK1, and iNOS. After five washes in blocking solution, blots were incubated with horseradish peroxidase-labeled anti-rabbit IgG or antimouse IgG (GE Healthcare) for 2 h at room temperature and were washed three times with Tris-buffered saline/Tween 20 (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 7.6 at 25°C). This was followed by three washes with Tris-buffered saline without 0.1% Tween 20 treated with ECL Plus reagents, and the protein of interest was visualized by exposure to Kodak BioMax XAR film (Fisher Scientific Co., Pittsburgh, PA).

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared from the cells as described previously (Schreiber et al., 1989). In brief, the cells were suspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% Nonidet P-40, 1 mM DTT, and 1 mM PMSF). The mixtures were centrifuged at 5000g for 30 s, and the cytosolic extract was separated. The nuclear pellet was washed with excess volume of buffer A and then resuspended in buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF). After incubating for 5 min at 4°C with...
rotation, the extracts were centrifuged (5000g, 1 min) and the supernatants were used for electrophoretic mobility shift assays (EMSA).

EMSA were performed by incubating nuclear extracts with 32P-radioabeled double-stranded oligonucleotide probes suspended in reaction buffer (12 mM HEPES, pH 7.9, 100 mM NaCl, 0.25 mM EDTA, 1 mM DTT, and 1 mM PMSF) at room temperature for 10 min. The protein-DNA complexes were electrophoresed using 4% nondenaturing polyacrylamide gels, dried, and exposed to X-ray films (Fisher Scientific Co) or to phosphorimaging screen (Cyclone Storage Phosphor System; PerkinElmer Life and Analytical Sciences). The -fold increase in the expression of the transcription factors was determined using background subtract. The NF-κB probe was a double-stranded oligonucleotide containing the consensus site for NF-κB binding: 5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′.

Protein Determination and Statistical Analyses. Saturation and competition curves for radioligand binding were performed using the Bradford protein assay (Bradford, 1976) and bovine serum albumin to prepare standard curves. Statistical analyses were performed by the analysis of variance and Dunnet's post hoc test.

Results
Characterization of A3AR in RAW 264.7 Cells. Radioligand binding experiments performed using [125I]AB-MECA indicated specific binding of the radioligand using theophylline (1 mM) to define nonspecific binding. This binding showed greater sensitivity to MRS1220, a selective antagonist of the A3AR, than to DPCPX and ZM241385, selective antagonists of the A1 and A2AAR, respectively (Fig. 1, A and B). These data indicated preferential binding of the radioligand to the A3AR and suggest that this radioligand would be useful for quantitating this receptor subtype. Saturation curves performed with increasing concentrations of the radioligand indicate $B_{max}$ and $K_d$ values of $68 \pm 14$ fmol/mg protein and $1.24 \pm 0.74$ nM, respectively (Fig. 1C). Expression of the A3AR on RAW 264.7 cells was further confirmed by immunocytochemistry using a polyclonal antibody. The A3AR appeared as red fluorescence under confocal microscopy using a krypton laser (Fig. 1D). The yellow fluorescence indicates nuclear staining of permeabilized cells (0.05% Triton X-100) using 0.5 μM Sytox (Invitrogen). No staining was observed when no primary antibody was added, indicating specificity of antibody staining. Furthermore, we observed increases in both A3AR immunostaining and radioligand binding following treatment of RAW 264.7 cells with LPS for 6 h, possibly implying labeling of the same protein using these two different techniques (data not shown). Additional studies also indicate low levels of A1AR and the A2AAR immunoreactivity in RAW 264.7 cells (data not shown).

Activation of the A3AR Inhibits LPS-Induced Release of TNF-α. To determine the effect of A3AR activation on LPS-stimulated TNF-α production in RAW 264.7 cells, dose-response curves were performed using different adenosine analogs. Basal TNF-α levels measurements performed by ELISA ranged from ~50 to 400 pg/ml, whereas levels attained following 6-h LPS exposure ranged from 9000 to 12,000 pg/ml. Figure 2A indicates inhibition of TNF-α production in cultures pretreated with either R-PIA or IB-MECA prior to the addition of LPS. IB-MECA, a selective agonist at the A3AR, demonstrated greater potency and maximal inhibition than that observed with R-PIA, a nonselective AR analog, indicating a role of the A3AR in mediating this action. Inhibition produced by the highest concentration of R-PIA and IB-MECA used (50 μM) were 55 ± 7 and 91 ± 7%, respectively. The IC$_{50}$ value for IB-MECA was 15.8 ± 1.3 μM.
\(\mu M\), whereas that for \(R\)-PIA could not be obtained because only partial inhibition of LPS-stimulated TNF-\(\alpha\) release was observed at the highest concentration of \(R\)-PIA used. The involvement of the A3AR in mediating inhibition of LPS-stimulated TNF-\(\alpha\) release was further supported by the observation that pretreatment of cultures with MRS1220 (1 \(\mu M\)) significantly reversed the inhibition produced by both \(R\)-PIA (50 \(\mu M\)) and 10 \(\mu M\) IB-MECA (\(p < 0.05\)) (Fig. 2B). In contrast, in cells pretreated with either 1 \(\mu M\) DPCPX or 1 \(\mu M\) ZM241385, the inhibitory action of \(R\)-PIA was unaffected. Additional studies were undertaken to determine whether selective A2AAR activation could inhibit LPS-stimulated TNF-\(\alpha\) release. Cells treated with 0.1, 1, and 10 \(\mu M\) CGS21680 showed LPS-stimulated TNF-\(\alpha\) levels, which were 88 \(\pm\) 7, 86 \(\pm\) 8, and 91 \(\pm\) 15\% of control, respectively, indicative of a lack of significant inhibitory effect of this receptor subtype. Taken together, these data strongly implicate the A3AR in mediating inhibition of LPS-stimulated TNF-\(\alpha\) release by \(R\)-PIA and IB-MECA.

Adenosine Analogs Inhibit LPS-Stimulated Intracellular Ca\(^{2+}\) Release. Previous studies have shown that LPS stimulates intracellular Ca\(^{2+}\) release in peritoneal macrophages (Letari et al., 1991), which is crucial for the stimulation of TNF-\(\alpha\) release (Chen et al., 2001). Therefore, we tested whether activation of the A\(_3\)AR suppresses LPS-mediated intracellular Ca\(^{2+}\) release. In these experiments, baseline fluorescence was collected for 2.5 min prior to the addition of LPS. Treatment of RAW 264.7 cells with LPS (3 \(\mu g/ml\)) produced a rapid rise in intracellular Ca\(^{2+}\) release, as measured by Fluo-4 AM fluorescence (Fig. 3A). The increase was immediate and reached a maximum level (~70\% increase) by 3.3 min (see graphical representation in Fig. 3B, top panel). The LPS-stimulated increase in intracellular Ca\(^{2+}\) release was attenuated when cultures were pretreated with 50 \(\mu M\) \(R\)-PIA and by 10 \(\mu M\) IB-MECA (Fig. 3A), suggesting that such a mechanism could account for the inhibition of TNF-\(\alpha\). Cells treated with BAPTA-AM (10 \(\mu M\)), a cell-permeable Ca\(^{2+}\) chelator, showed an approximately 4-fold reduction in LPS-stimulated TNF-\(\alpha\) release (Fig. 3C), which was greater than that observed with \(R\)-PIA (50 \(\mu M\)). In all of these experiments, fluorescence intensities were estimated over a 3-min period following the addition of LPS. In cultures pretreated with BAPTA, \(R\)-PIA was able to further inhibit TNF-\(\alpha\) release. This suggests that modulation of intracellular Ca\(^{2+}\) release by adenosine analogs contributes significantly to inhibition of TNF-\(\alpha\) release, even though it

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**Fig. 3.** Activation of the A\(_3\)AR inhibits LPS-stimulated intracellular Ca\(^{2+}\) release. RAW 264.7 cells were cultured on coverslips, washed with physiological saline, and loaded with the Ca\(^{2+}\) indicator dye Fluo-4 AM (5 \(\mu M\)) for 45 min. Cells were then pretreated with adenosine deaminase (1 U/ml) and with either vehicle, \(R\)-PIA (50 \(\mu M\)) or IB-MECA (10 \(\mu M\)), for 5 min followed by LPS (3 \(\mu g/ml\)) for the times indicated. A, peak fluorescence detected by confocal microscopy using an argon laser (488 nm) at 250 s following the addition of LPS. Data show a 200-fold magnification of the cells. The increase in fluorescence over time following addition of LPS is depicted in B. The figures show the mean fluorescence obtained from counting 20 cells per microscope field over a 6-min period. C, LPS-stimulated TNF-\(\alpha\) release was inhibited by chelating intracellular Ca\(^{2+}\) with BAPTA-AM (10 \(\mu M\)) and extracellular Ca\(^{2+}\) with EGTA (1 mM). The data represent the mean \(\pm\) S.E.M. of four determinations.
might not totally explain it. Additional experiments were performed to examine whether inhibition of Ca\(^{2+}\) entry through the plasma membrane by adenosine analogs could also contribute to the inhibition of TNF-α release. Treatment of cultures with 1 mM EGTA reduced LPS-stimulated TNF-α production and abrogated the inhibitory action of R-PIA (Fig. 3C). Similarly, pretreatment of RAW 264.7 cells with the Ca\(^{2+}\) channel blocker nifedipine (10 μM) produced an ~30% reduction in LPS-stimulated intracellular TNF-α release (data not shown). These data suggest that a reduction in intracellular Ca\(^{2+}\) availability, either through inhibition of intracellular Ca\(^{2+}\) release or plasma membrane Ca\(^{2+}\) entry, could account fully for the reduction in TNF-α release produced following activation of the A\(_3\)AR in RAW 264.7 cells.

**Involvement of the Mitogen-Activated Protein Kinase-1 and NF-κB Pathways in LPS-Stimulated TNF-α Production in RAW 264.7 Cells.** Previous studies indicate that TLR activate a common signaling pathway leading to the activation of NF-κB and MAPK (Aderem and Ulevitch, 2000). Therefore, we determined whether these downstream effectors also contribute to LPS-stimulated TNF-α production in RAW 264.7 cells and determined the level of involvement of each of these pathways. Pretreatment of RAW 264.7 cells with PD098059, a specific inhibitor of mitogen-activated protein kinase-1, produced a dose-dependent inhibition of LPS-stimulated TNF-α production, with the highest dose of this agent (50 μM) producing a 54.9 ± 3.7% inhibition (Fig. 4). Furthermore, cells treated with the pyrrolidine derivative PDTC, an inhibitor of NF-κB, demonstrated significantly reduced induction of TNF-α (83 ± 5% reduction), indicating a role of this pathway in mediating TNF-α production in these cells.

**A\(_3\)AR Decreased the Phosphorylation of ERK1/2 and NF-κB Activation.** Based on the observation that ERK and NF-κB pathways contribute to LPS-stimulated TNF-α production in RAW 264.7 cells, additional experiments were performed to directly evaluate whether activation of the A\(_3\)AR decreases LPS-stimulated activation of MAPK. In cultures stimulated with LPS, we observed a 2.4-fold increase in ERK1/2 phosphorylation. Cells treated with IB-MECA (10 μM) during LPS administration demonstrated an almost complete abolition of LPS-stimulated ERK phosphorylation (Fig. 5). This inhibitory action was completely reversed by the addition of MRS1220 (1 μM) to cultures prior to IB-MECA, indicating that inhibition of ERK1/2 phosphorylation was mediated via the A\(_3\)AR. Furthermore, reversed by MRS1220 indicated that the inhibition of ERK1/2 phosphorylation was not a result of toxicity to the cells produced by IB-MECA. The addition of BAPTA (10 μM) to these cells to chelate intracellular Ca\(^{2+}\) significantly reduced LPS-stimulated ERK1/2 phosphorylation, also implying a role of intracellular Ca\(^{2+}\) release in ERK1/2 activation in these cells (Fig. 5). However, significant LPS-stimulated ERK1/2 phosphorylation was evident even in the presence of BAPTA, suggesting that other factors in addition to Ca\(^{2+}\) are involved in LPS-dependent activation of ERK1/2. Cells treated with 50 μM PD098059 prior to the addition of LPS showed a 69 ± 7% reduction in ERK1/2 phosphorylation, statistically equivalent to that observed with IB-MECA (data not shown).

To determine whether activation of the A\(_3\)AR reduces NF-κB activity, RAW 264.7 cells were pretreated with IB-MECA (10 μM) prior to the exposure of LPS for 30 min. Cytosolic extracts were prepared and examined for the degradation of IκB-α, indicative of NF-κB activation. As shown in Fig. 6, A and B, LPS induced significant degradation of IκB-α within 30 min, which was reversed by IB-MECA. The response of IB-MECA was attenuated by MRS1220 (1 μM), indicating a role of A\(_3\)AR in mediating inhibition of NF-κB. Reversal by MRS1220 also indicates that the inhibition of IκB-α degradation by IB-MECA was not a result of toxicity to the cells. Interestingly, incubation of cells with BAPTA-AM for 30 min also attenuated the LPS-stimulated NF-κB activation, implicating intracellular Ca\(^{2+}\) release in the activation process.

The activation of NF-κB by LPS and subsequent inhibition by IB-MECA were confirmed by EMSA. These data indicate a 3 to 5-fold increase in NF-κB activity induced by a 30-min exposure to LPS, which was reduced in a dose-dependent fashion by IB-MECA (Fig. 6C). These data indicate that
NF-κB is a major downstream target of inhibition by the A3AR and that this mechanism serves as a likely mediator of the anti-inflammatory effects of adenosine analogs.

**Activation of the A3AR Inhibits LPS-Stimulated iNOS Expression.** Activation of NF-κB (Barnes and Karin, 1997) and MAPK (Dong et al., 2002) plays an important role in mediating LPS-stimulated expression of iNOS in macrophage. To determine whether A3AR activation regulates iNOS protein expression, cells were treated with LPS in the absence and presence of adenosine analogs. LPS treatment significantly increased the iNOS protein expression (Fig. 7A), which was reduced by activation of the A3AR. Pretreatment with MRS1220 (1 μM) attenuated the inhibition of iNOS expression produced by IB-MECA, indicative of a role of the A3AR in mediating this response. Similarly, the addition of R-PIA (50 μM) to cultures produced significant inhibition of iNOS protein expression, even though this small level of inhibition was less than that observed for IB-MECA (Fig. 7B).

**Discussion**

The results of this study support a role of the A3AR in suppressing LPS-stimulated TNF-α release in RAW 264.7 cells. The mechanism underlying this inhibition in RAW 264.7 cells clearly involves a reduction in cytosolic Ca^{2+} mediated by inhibition of both intracellular Ca^{2+} release and plasma membrane Ca^{2+} entry. Furthermore, we show that intracellular Ca^{2+} release is critical for LPS activation of NF-κB and the ERK1/2 pathways, both of which are crucial for the regulation of TNF-α and iNOS gene expression. Thus, A3AR activation disrupts a critical step, which alters LPS signal transduction pathways, leading to reductions in TNF-α and iNOS production.

A rise in intracellular Ca^{2+} accompanies LPS stimulation of peritoneal macrophages and RAW 264.7 cells (Kim et al., 2004) and is crucial for the stimulation of TNF-α release in murine peritoneal macrophage (Chen et al., 2001). These investigators showed that the addition of LPS with thapsigargin, an inhibitor of the Ca^{2+}-ATPase pump in the endoplasmic reticulum, produced a more efficient transcription of TNF-α mRNA, whereas chelation of intracellular Ca^{2+} abolished the Ca^{2+}-dependent TNF-α production. LPS also elevates the basal levels of intracellular Ca^{2+} in brain microglia (Hoffmann et al., 2003). Our data support a role of Ca^{2+}, both from intracellular and extracellular sources, in regulating LPS-stimulated TNF-α release, since inhibition of release was observed by both BAPTA-AM and EGTA and a combination of BAPTA and EGTA produced an almost complete loss of TNF-α production, suggesting that Ca^{2+} is the principal second messenger regulating synthesis and release of this inflammatory cytokine.

The target(s) of A3AR, which inhibit(s) the rise in intracellular Ca^{2+}, is unclear at present. Although the inhibitory effect of R-PIA was reduced by BAPTA, it was not completely abrogated. This suggests that A3AR might be coupled to both inhibition of intracellular Ca^{2+} release stores and inhibition of plasma membrane Ca^{2+} entry via voltage-sensitive Ca^{2+} channels and/or Ca^{2+}-release-activated Ca^{2+} channels. Indirect support for a role of these plasma membrane Ca^{2+} channels sites as targets for inhibition by A3AR agonists is provided by the observation that depletion of extracellular Ca^{2+} with EGTA (which should render these channels functionally inactive) abrogated the LPS-stimulated release of TNF-α. Additionally, LPS-stimulated cells treated with
BAPTA-AM showed reduced levels of IkB-α degradation, suggesting that Ca²⁺ acts either at the level of or upstream of IkB-α phosphorylation.

The present data strongly support the contention that suppression of LPS-stimulated TNF-α production via the A₃AR in RAW 264.7 cells involves inhibition of NF-κB. Several pieces of evidence support the involvement of intracellular Ca²⁺ release in the activation of NF-κB. Activation of NF-κB by oxidant stress in the endoplasmic reticulum stress is dependent on intracellular Ca²⁺ release (Pahl and Baueerle, 1996). Oxidant challenge in Jurkat T cells and Wurzburg cells increases Ca²⁺ from intracellular stores which resulted in NF-κB activation (Sen and Packer, 1996). Furthermore, increase in intracellular Ca²⁺ subsequent to glutamate receptor activation promotes NF-κB activation (Mattson and Camandola, 2001). In addition, Ca²⁺, along with protein kinase C, has also been implicated in the activation of the NF-κB in pancreatic lobules by the cholecystokinin analog caerulein (Tando et al., 1999). One mechanism by which elevated intracellular Ca²⁺ couples to NF-κB activation seems unique. Studies in dendritic cells showed that LPS-stimulated NF-κB activation is mediated by a Ca²⁺-dependent and cyclosporin A-sensitive pathway, implicating the phosphatase calcineurin in the activation process (Lyakh et al., 2000). Furthermore, activation of NF-κB in pancreatic lobules by the caerulein, a cholecystokinin analog (Tando et al., 1999), is also inhibited by cyclosporin A. Thus, it is possible that inhibition of intracellular Ca²⁺ release and calcineurin could account for the reduction in LPS-mediated activation of NF-κB by the A₃AR.

Recent studies claimed that activation of the A₂₃₅AR in primary mouse macrophages by NECA, a nonselective agonist of this receptor, decreased TNF-α production by a NF-κB-independent process (Pinhal-Einfield et al., 2003). However, the identity of the AR subtype was based primarily on this ligand, without the use of an appropriate antagonist. Similar studies by this group (Németh et al., 2003) have shown that adenosine inhibits LPS-stimulated TNF-α release by a mechanism independent of NF-κB activation and by an AR subtype, which was not clearly defined. Certainly, these investigators observed a significant reduction in LPS-stimulated TNF-α release in these cells with IB-MECA, a selective A₃AR agonist (Németh et al., 2003). The reason for the discrepancy between our data and those described above concerning NF-κB as a target for AR interaction is not clear. Our data, obtained using selective agonists and antagonists, clearly indicate a principal role of the A₃AR in suppressing LPS-stimulated TNF-α release and NF-κB activation in RAW 264.7 cells.

One problem with ascribing a role of the A₃AR in inhibiting TNF-α release in RAW 264.7 cells is that the concentrations of IB-MECA needed to produce significant inhibition far exceed the Kᵣ for interaction at the A₃AR (generally in the nanomolar range). Although the reason for this discrepancy is not clear, it could reflect difference in A₃AR-G protein coupling and/or post receptor signaling processes in this cell type. In general, studies with the RAW264.7 line have used high micromolar concentrations of adenosine analogs to effect functional responses (Haskó et al., 1996; Min et al., 2000; Németh et al., 2003). Other studies have utilized these high concentrations of agonists to elicit functional responses attributed to A₃AR activation in other cell types. For example, Sajjadi et al. (1996) have shown inhibition of LPS-stimulated TNF-α release in human macrophage using IB-MECA in the micromolar range. Similar micromolar concentrations of IB-MECA were shown to suppress liposaccharide-mediated respiratory burst in cultured human monocytes (Thiele et al., 2004).

In addition to NF-κB, activation of the A₃AR suppresses LPS activation of the ERK pathway. We showed that this pathway is important for mediating LPS stimulation of TNF-α production, because treatment with PD98059, an inhibitor of this pathway, resulted in partial inhibition of the LPS response. This pathway could also serve as an additional site at which the inhibitory action of the A₃AR is manifested. The mechanism underlying LPS-activated ERK phosphorylation also seems to be dependent on intracellular Ca²⁺ release, because treatment of cells with BAPTA significantly reduced LPS-stimulated ERK1/2 phosphorylation. Thus, by modulating intracellular Ca²⁺ release, the A₃AR can regulate the LPS activation of both the ERK1/2 and NF-κB pathways in RAW264.7 macrophages coupled to TNF-α production. Unlike the data presented here, activation of the A₃AR in transfected Chinese hamster ovary cells by the agonist NECA stimulated ERK1/2 phosphorylation, independent of Ca²⁺ mobilization and protein kinase C activation (Schulte and Fredholm, 2000, 2002) but involving the phosphatidylinositol 3-kinase/Ras signaling pathway.

The induction of TNF-α by LPS in macrophages is mediated by transcriptional (Raabe et al., 1998), post-transcriptional (Jarrous et al., 1996), translational (Han et al., 1990), and post-translational mechanisms (Ribeiro et al., 1996). In LPS-stimulated RAW 264.7 cells, the large increases in the levels of secreted TNF-α were primarily due to the activation of transcription (Raabe et al., 1998). Seven transcription binding sites have been identified in the TNF-α promoter region, and it seems that full activation of TNF-α relies on recruitment of NF-κB, Egr-1, and c-Jun (Yao et al., 1997; Tsai et al., 2000). For example, it has been demonstrated that LPS-stimulated ERK1/2 activation leads to increased binding of Erg-1 to the TNF-α promoter in RAW 264.7 cells (Shi et al., 2002).

One of the best potential therapeutic applications of these findings is the treatment of sepsis, the most common cause of deaths in intensive care units (Angus et al., 2001). There is indirect evidence that intracellular Ca²⁺ is elevated in sepsis, which contributes to increased cellular injury (Song et al., 1993). Gabexate mesylate, a synthetic protease inhibitor, has proven effective in treating sepsis-associated symptoms, presumably by inhibiting the signaling pathways leading to TNF-α production (Yuksel et al., 2003). In animal models of septic shock, adenosine confers an anti-inflammatory action (Firestein et al., 1994), presumably by inhibiting similar pathways described for LPS. Perioperative infusion of adenosine provides effective control of surgical pain in human without producing major side effects (Fukunaga et al., 2003), suggesting that this agent could be similarly administered to treat sepsis.

In summary, our data provide novel information concerning the mechanism of inhibition of LPS-stimulated TNF-α release in RAW 264.7 cells by adenosine analogs. The data demonstrate a principal role of the A₃AR in mediating the anti-inflammatory action of adenosine and indicate that this action is mediated, in part, by inhibiting the rise in intracel-
ular Ca\(^{2+}\) and the activation of NF-κB and the ERK pathways following LPS challenge.

**References**


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