Double-stranded RNA-induced Inducible Nitric-oxide Synthase Expression and Interleukin-1 Release by Murine Macrophages Requires NF-κB Activation

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The effects of double-stranded RNA (synthetic polynucleosinic-polyribonucleic acid; poly(I-C)) on macrophage expression of inducible nitric-oxide synthase (iNOS), production of nitric oxide, and release of interleukin-1 (IL-1) were investigated. Individually, poly(I-C), interferon-γ (IFN-γ), and lipopolysaccharide (LPS) stimulate nitrite production and iNOS expression by RAW 264.7 cells. In combination, the effects of poly(I-C) + IFN-γ are additive, while poly(I-C) does not further potentiate LPS-induced nitrite production. These results suggest that poly(I-C) and LPS may stimulate iNOS expression by similar signaling pathways, which may be independent of pathways activated by IFN-γ. LPS-induced iNOS expression is associated with the activation of NF-κB. We show that inhibition of NF-κB by pyrrolidinedithiocarbamate prevents poly(I-C) + IFN-γ, poly(I-C) + LPS-, and LPS-induced iNOS expression, nitrite production and iκB degradation by RAW 264.7 cells. The effects of poly(I-C) on iNOS expression appear to be cell-type specific. Poly(I-C), alone or in combination with IFN-γ, does not stimulate, nor does poly(I-C) potentiate, IL-1-induced nitrite production by rat insulinoma RINm5F cells. In addition, we show that the combination of poly(I-C) + IFN-γ stimulates iNOS expression, nitrite production, iκB degradation, and the release of IL-1 by primary mouse macrophages, and these effects are prevented by pyrrolidinedithiocarbamate. These findings indicate that double-stranded RNA, in the presence of IFN-γ, is a potent activator of macrophages, stimulating iNOS expression, nitrite production, and IL-1 release by a mechanism which requires the activation of NF-κB.

Nitric oxide (NO) is the product of the five-electron oxidation of l-arginine to l-citrulline catalyzed by the enzyme nitric-oxide synthase (NOS) (1). Three isoforms of NOS have been cloned and characterized: endothelial NOS (eNOS or NOSIII), neuronal NOS (nNOS or NOSI), and inducible NOS (iNOS or NOSII) (2–4). Collectively, eNOS and nNOS are known as cNOS because their enzymatic activity is regulated by Ca²⁺ and because these isoforms are constitutively expressed. Nitric oxide, produced in low levels by cNOS isoforms, functions as a signaling molecule associated with diverse biological processes including the regulation of vascular tone and neuronal signaling (4–6). Nitric oxide, produced in large quantities following induction of iNOS by cytokines or endotoxin, can have cytotoxic or cytostatic effects on target cells (2, 7) and has recently been implicated as an effector molecule that participates in antiviral responses (8, 9).

Viral infection has been shown to stimulate NO production by iNOS in several cell types including mixed glial cell cultures, lymphocytes and monocytes/macrophages (10–13). Karupiah et al. (14) have shown NO production is required for IFN-γ inhibition of ectromelia, vaccinia, and herpesvirus replication in mouse macrophages. Also, Bukrinsky et al. (15) have shown that infection of human monocytes with human immunodeficiency virus type 1 stimulates iNOS expression and nitric oxide production. Although viral infection stimulates iNOS expression and nitric oxide production, the mechanism by which viral infection stimulates iNOS expression is unknown.

NF-κB appears to play a primary role in the transcriptional regulation of iNOS gene expression by macrophages (16, 17). In unstimulated cells, NF-κB is found as an inactive heterodimer of p50/p65 subunits bound to the NF-κB inhibitor protein, IκB. Upon stimulation, IκB is phosphorylated on specific serine residues which targets IκB for degradation in a ubiquitin-dependent manner (18). The antioxidant inhibitors of NF-κB activation, pyrrolidinedithiocarbamate (PDTC) and diethyldithiocarbamic acid, prevent lipopolysaccharide (LPS)- and LPS + IFN-γ-induced iNOS expression and nitrite production by RAW 264.7 cells (17), indicating that NF-κB participates in LPS- and LPS + IFN-γ-induced iNOS expression.

The active component of a viral infection that stimulates antiviral activities appears to be double-stranded RNA (dsRNA), which accumulates during the replication of many viruses. The purpose of this study was to determine if the activation of macrophages by dsRNA results in iNOS expression, nitric oxide production, and IL-1 release, and if these events are dependent on NF-κB activation. We show that dsRNA (in the form of poly(I-C)), in combination with IFN-γ, is a potent activator of murine macrophages, stimulating iNOS expression, nitric oxide production, and IL-1 release. Furthermore, we show that polynucleosinic-polyribonucleic acid (poly(I-C))-induced iNOS expression and IL-1 release are associated with the activation of NF-κB. These studies provide direct support for double-stranded RNA as one effector molecule that medi-
ates macrophage activation in an NF-κB-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials and Animals—**Mouse macrophage RAW 264.7 and rat insulinoma RINm5F cells were obtained from Washington University Tissue Culture Support Center. RPMI medium 1640 containing 1× L-glutamine, CMRL-1066 tissue culture medium, L-glutamine, penicillin, streptomycin, and mouse and rat recombinant IFN-γ were from Life Technologies, Inc. Fetal calf serum was obtained from Hyclone (Logan, UT). Male CD-1 mice (20–24 g) were purchased from Harlan (Indianapolis, IN). Aminoguanidine (AG) hemisulfate, LPS (serotype 0111:B4), poly(I-C), and PDTC were from Sigma. [γ-32P]dCTP and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Corp. NF-κB consensus oligonucleotide and rabbit anti-κB-a and κB-β antisera were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit antisera specific for the C-terminal 27 amino acids of mouse macrophage iNOS was a gift from Dr. Thomas Misko (G. D. Searle, St. Louis, MO). iNOS and cyclophilin cDNAs were gifts from Dr. Steve Carroll (Department of Pathology, University of Alabama-Birmingham, AL), respectively. All other reagents were from commercially available sources.

**CD-1 Mouse Peritoneal Macrophage Isolation and Cell Culture—**Peritoneal macrophages (peritoneal exudate cells, PEC) were isolated from male CD-1 mice by lavage as described previously (19). Following isolation, the cells were plated at a concentration of 400,000 cells/400 μl complete CMRL-1066 (CMRL-1066 containing 2 μg/ml L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) in 24-well microtiter plates and incubated for 3 h under an atmosphere of 95% air and 5% CO2 at 37 °C. Nonadherent cells were washed twice with ice-cold 0.1 M phosphate-buffered saline, resuspended in 400 μl of buffer I (10 mM HEPES, pH 7.8, 5 mM MgCl2, 10 mM KCL, 1 mM ZnCl2, 0.2 mM EDTA, 1 mM Na2VO4, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μM of each of the following: leupeptin, antipain, aprotinin, and pepstatin A) and incubated on ice for 10 min. Nuclei were then isolated, the cells were plated at a concentration of 400,000 cells/400 μl of culture medium with 50 μg/ml poly(I-C) (Boehringer Mannheim) in a buffer containing 10 mM HEPES (pH 7.8), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol and were incubated at 37 °C for 20 min. Nuclear extracts were separated by centrifugation (13,500 rpm, 4 °C, 15 min), and the nuclear protein extract was used for gel shift analysis. Protein concentration was determined by the BCA protein assay according to manufacturer's instructions (Pierce).

**Gel Shift Analysis—**Gel shift analysis of nuclear extracts was performed as described previously (24, 25). The probe consisted of a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB (5′-AGT GTA GGG GAC TTT CCC AGG C-3′; Santa Cruz) end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). Typical binding reactions consisted of 10 μg of nuclear extract, 0.5 ng of DNA probe, 2 μg/ml poly(dI-C) (Boehringer Mannheim) in a buffer containing 20 mM HEPES (pH 7.8), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol and were incubated at 37 °C for 20 min. Binding reactions were separated on 4% Tris-glycine nondenaturing polyacrylamide gels in a 2× Tris-glycine (0.05 M Tris-HCl, pH 8.3, 0.38 M glycine, 2 mM EDTA) buffer system (26). The gels were transferred to Whatman paper, dried, and subjected to autoradiography.

**Nitrite and IFN-γ Determination—**Nitrite production was determined by mixing 50 μl of culture medium with 50 μl of Griess reagent (27). The absorbance at 540 nm was measured, and nitrite concentrations were calculated from a sodium nitrite standard curve. IL-1 release from mouse PEC was performed using the RINm5F cell bioassay as described by Hill et al. (28).

**RESULTS**

**Effects of Poly(I-C), IFN-γ, and LPS on Nitrite Production and iNOS mRNA and Protein Expression by RAW 264.7 Cells—**LPS and IFN-γ alone will activate mouse macrophage RAW 264.7 cells, to express iNOS and produce nitric oxide; however, maximal production of nitric oxide occurs in response to a combination of LPS + IFN-γ. To determine if dsRNA stimulates macrophage activation, the effects of the synthetic dsRNA molecule, poly(I-C), alone or in combination with LPS and IFN-γ on nitrite production by RAW 264.7 cells were examined. As shown in Fig. 1a, a 24-h incubation of RAW 264.7 cells with either poly(I-C) (100 μg/ml) or IFN-γ (150 units/ml) stimulates a 9– and 6-fold increase in nitrite production, respectively. While poly(I-C) does not appear to further increase the level of nitrite produced in response to LPS, in combination with IFN-γ, poly(I-C) stimulates an over 20-fold increase in nitrite production by RAW 264.7 cells. The levels of nitrite production in response to poly(I-C) + IFN-γ are similar in magnitude to the effects of IFN-γ + LPS on RAW 264.7 cell nitrite production. AG, a selective inhibitor of iNOS (29), com-
Nk-dependent Macrophage Activation by Double-stranded RNA

**Fig. 1.** Effects of poly(I-C), IFN-γ, and LPS on nitrite production, iNOS mRNA and protein expression by RAW 264.7 cells. (a) and (b), RAW 264.7 cells (400,000 cells/400 μl complete CMRL) were incubated for 24 h with the indicated concentrations of poly(I-C), LPS, and IFN-γ. Nitrite production was determined on the culture medium as described under “Experimental Procedures.” (c), RAW 264.7 cells (10 × 10^6 cells/3 ml of complete CMRL-1066) were cultured for 6 h at 37°C with the indicated concentrations of poly(I-C), mouse IFN-γ, and/or LPS. Total RNA was isolated and probed for iNOS and cyclophilin by Northern analysis as stated under “Experimental Procedures.” (d), RAW 264.7 cells (400,000 cells/400 μl of complete CMRL) were incubated for 24 h with the indicated concentrations of poly(I-C), LPS, and IFN-γ. The cells were isolated, and the expression of iNOS was determined by Western blot analysis as described under “Experimental Procedures.” Results for nitrite are the average ± S.E. of five independent experiments, and iNOS mRNA and protein expression are representative of two and three independent experiments, respectively.
IFN-γ (150 units/ml), LPS (10 μg/ml) and PDTC (100 μM) were then added, and the cells were cultured for 24 h. As shown in Fig. 2a, PDTC completely prevents poly(I-C), poly(I-C) + IFN-γ, LPS-, and LPS + poly(I-C)-induced nitrite production by RAW 264.7 cells. Consistent with its inhibitory effects on nitrite production, PDTC also inhibits poly(I-C) (data not shown), poly(I-C) + IFN-γ, LPS-, and LPS + poly(I-C)-induced iNOS protein expression (Fig. 2b). These findings provide evidence that poly(I-C) (in the presence or absence of IFN-γ) stimulates iNOS expression by a mechanism that is associated with the activation of NF-κB.

To determine if dsRNA activates NF-κB, we have evaluated the effects of poly(I-C) on NF-κB nuclear localization by gel shift analysis. As shown in Fig. 2c, treatment of RAW 264.7 cells for 30 min with poly(I-C) stimulates the nuclear localization of NF-κB, as evidenced by the reduced mobility of the DNA probe containing the consensus sequence for NF-κB binding. The stimulatory effects of poly(I-C) are similar to the effects of LPS on NF-κB activation by RAW 264.7 cells, and co-incubation of RAW 264.7 cells with IFN-γ or LPS does not further potentiate poly(I-C)-induced NF-κB activation. As controls for the gel shift assays, excess poly[d(I-C)] and cold NF-κB oligonucleotide inhibit the formation of the NF-κB-probe complex, and inclusion of antiserum specific for the p50 and p65 components of NF-κB in the binding reactions results in a further reduction in the mobility (supershift) of the NF-κB complex (data not shown).

To further examine the role of poly(I-C) in NF-κB activation, the effects of poly(I-C), alone and in combination with LPS and IFN-γ, on IkB degradation was examined. As shown in Fig. 2d, treatment of RAW 264.7 cells for 30 min with 100 μg/ml poly(I-C) results in the degradation of greater than 50% of IkB-α. IkB-α degradation is prevented by treatment of RAW 264.7 cells for 30 min with PDTC prior to the addition of poly(I-C). In combination, poly(I-C) + IFN-γ stimulate IkB-α degradation to levels similar in magnitude to the individual effects of poly(I-C), LPS, and LPS + poly(I-C), and this effect is prevented by PDTC. Also, PDTC inhibits LPS- and LPS + poly(I-C)-induced IkB-α degradation. We have also examined the effects of poly(I-C), LPS, and IFN-γ, alone and in combination, on IkB-β degradation and have obtained nearly identical results to those shown in Fig. 2d for IkB-α (data not shown). These findings indicate that poly(I-C) stimulates NF-κB nuclear localization by a mechanism that is associated with the degradation of IkB.

Effects of Poly(I-C) on Nitrite Production by RINm5F Cells—

The insulinoma RINm5F cell line represents a homogenous population of pancreatic islet β-cells that express iNOS and produce nitric oxide in response to IL-1 (31). In addition, IL-1-induced nitric oxide production by RINm5F cells appears to require the activation of NF-κB as PDTC and diethyldithiocarbamic acid inhibit both NF-κB activation and iNOS expression (25, 32). Recently, we have shown that IFN-γ potentiates IL-1-induced iNOS expression and nitrite production by RINm5F cells at concentrations of IL-1 that alone do not stimulate iNOS expression (20). Since poly(I-C)-induced iNOS expression and nitrite production by RAW 264.7 cells appears to require the activation of NF-κB, and IL-1-induced iNOS expression by RINm5F cells also requires NF-κB activation, the effects of poly(I-C), alone and in combination with IL-1 and IFN-γ, on nitrite production by RINm5F cells were examined (Fig. 3). Treatment of RINm5F cells with IL-1 stimulates a 20-fold increase in nitrite production following a 24-h incubation. Alone, poly(I-C) does not stimulate nitrite production, nor does it further potentiate IL-1-induced nitrite production by
FIG. 3. **Effects of poly(I-C) and IL-1β on nitrite formation by RINm5F cells.** RINm5F cells (400,000 cells/400 µl of complete CMRL) were incubated for 24 h with the indicated concentrations of poly(I-C), LPS, and IFN-γ, and nitrite production was determined on the culture supernatants as described under “Experimental Procedures.” Results are the average ± S.E. of four independent experiments containing three replicates/condition.

RINm5F cells. Individually, neither IFN-γ (150 units/ml) nor 0.1 units/ml IL-1 stimulate nitrite production by RINm5F cells; however, in combination these two cytokines stimulate the production of nitrite to levels nearly identical to the levels stimulated by maximal concentrations of IL-1 (1 unit/ml). In combination with either IFN-γ or submaximal concentrations of IL-1 (0.1 unit/ml), poly(I-C) does not stimulate nitrite production by RINm5F cells. Poly(I-C) also does not further potentiate IL-1 (submaximal or maximal concentrations) + IFN-γ-induced nitrite production (data not shown). These findings suggest that the actions of poly(I-C) on iNOS expression and nitrite production may be cell-type specific.

**Activation of Peritoneal Macrophages by Poly(I-C)—**In contrast to RAW 264.7 cells, resident mouse macrophages require a combination of two signals (e.g. IFN-γ + LPS) for iNOS expression and nitric oxide production. The effects of dsRNA on primary macrophage activation was examined by incubating mouse PEC with poly(I-C) in the presence or absence of IFN-γ and LPS. Alone, neither poly(I-C), IFN-γ, nor LPS stimulate nitrite production or iNOS expression by mouse PEC (Figs. 4, a and b, respectively). However, in combination, poly(I-C) + IFN-γ stimulate the expression of iNOS and the production of nitrite to levels comparable to the effects of IFN-γ + LPS. The production of nitrite in response to poly(I-C) + IFN-γ, or LPS + IFN-γ is completely prevented by the iNOS inhibitor AG. Importantly, the combination of poly(I-C) + LPS does not stimulate iNOS expression or nitrite production by PEC. This finding is consistent with the inability of poly(I-C) to potentiate LPS-induced nitrite production by RAW 264.7 cells. These findings indicate that a combination of poly(I-C) and IFN-γ activates primary macrophages stimulating iNOS expression and nitrite production.

**Poly(I-C) + IFN-γ Stimulate IL-1 Release by Primary Macrophages in an NF-κB-dependent Manner—**Macrophage activation is characterized by the release of high levels of the cytokine IL-1. Using the RINm5F cell bioassay (28) we have examined the effects of poly(I-C) on IL-1 release by PEC, and whether poly(I-C)-induced IL-1 release requires NF-κB activation. Alone, poly(I-C) (100 µg/ml), IFN-γ (150 units/ml), or LPS (10 µg/ml) did not stimulate IL-1 release by PEC following a 24-h incubation (Fig. 5a). However, the combinations of poly(I-C) + IFN-γ and LPS + IFN-γ stimulate the release of IL-1 to levels that are over 10-fold higher than that of control PEC. Preincubation of mouse PEC for 30 min with 100 µM PDTC completely inhibits poly(I-C) + IFN-γ-induced IL-1 release, implicating a role for NF-κB activation in IL-1 release by PEC.

To further examine the role of poly(I-C) on NF-κB activation in mouse PEC, the effects of poly(I-C), alone and in combination with LPS and IFN-γ, on IκB-α degradation was examined. Similarly to RAW 264.7 cells, poly(I-C), LPS, poly(I-C) + IFN-γ and LPS + poly(I-C) induce the degradation of greater than 50% of IκB-α, and these effects are prevented by PDTC (Fig. 5b). PDTC also prevents poly(I-C) + IFN-γ- and LPS + IFN-γ-induced nitrite formation by CD-1 mouse PEC (data not shown). These findings implicate IκB degradation and NF-κB activation in poly(I-C) + IFN-γ-induced nitrite production and IL-1 release by mouse macrophages.

**DISCUSSION**

One cellular response to viral infection is the expression of iNOS and the increased production of nitric oxide (8, 9). The mechanism by which viral infection stimulates iNOS expression is unknown. The active component of viral infection appears to be dsRNA, which accumulates during replication of many viruses (33). It was first demonstrated in 1971 that treatment with dsRNA (poly(I-C)) renders macrophages cytotoxic to target cells in a manner similar to the actions of endotoxin and lipid A (34). dsRNA-induced inhibition of protein translation and expression of type I interferons are associated with the antiviral activity in infected cells (33, 35). In the current study, the effects of dsRNA on macrophage activation and the mechanism by which dsRNA activates macrophages have been examined. Treatment of RAW 264.7 cells with poly(I-C) stimulates iNOS expression and nitrite production. IFN-γ potentiates poly(I-C)-induced iNOS expression and nitrite production, while LPS does not further potentiate poly(I-C)-induced nitrite production by RAW 264.7 cells. Alone,
poly(I-C) does not stimulate iNOS expression by primary macrophages (PEC); however, in combination with IFN-γ, poly(I-C) stimulates iNOS expression and high levels of nitrite production. In addition, poly(I-C) + IFN-γ stimulate IL-1 release by mouse PEC. Similar to the inability of LPS to enhance poly(I-C)-induced nitrite production by RAW 264.7 cells, poly(I-C), in combination with LPS, does not stimulate iNOS expression, nitrite production, or IL-1 release by mouse PEC.

Poly(I-C) appears to stimulate iNOS expression by a mechanism similar to that induced by LPS because the effects of LPS and poly(I-C) are not additive, while RAW 264.7 cell expression, nitrite production, or IL-1 release by mouse PEC.

The effects of poly(I-C) on iNOS expression appear to be cell-type specific. While poly(I-C), alone and in combination with IFN-γ, stimulates iNOS expression by RAW 264.7 cells, poly(I-C), alone or in combination with IFN-γ, does not induce nor does it enhance IL-1-induced nitrite production by the pancreatic beta cell line RINm5F. Previous studies have shown that IL-1 stimulates high levels of iNOS expression and nitric oxide production by RINm5F cells, and the actions of IL-1 are potentiated by IFN-γ (20, 31). IL-1-induced iNOS expression by RINm5F cells is associated with IkB degradation and NF-κB nuclear localization (25, 32). Poly(I-C) stimulates IkB degradation in RINm5F cells (data not shown); however, poly(I-C) does not stimulate iNOS expression or nitrite production. These results indicate that other transcriptional regulators in addition to NF-κB are required for IL-1-induced iNOS expression by RINm5F cells.

The antiviral response generated in infected cells includes the expression of type 1 interferons, and the inhibition of protein synthesis due to the phosphorylation of elongation factor 2α (33, 35). A number of studies have implicated a dsRNA intermediate as the activator of the antiviral response in infected cells (34, 38, 39). Also, viral infection stimulates iNOS expression by target cells, and nitric oxide appears to participate in the inhibition of viral replication (8–15). In this study, evidence is presented which implicates NF-κB as one transcriptional regulator that is activated and participates in the antiviral response triggered by treatment of macrophages with dsRNA. In addition, IL-1, released following dsRNA treatment (in the presence of IFN-γ), may also play a primary role in the antiviral activities of macrophages.

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