Interactive report

Muramyl dipeptide potentiates cytokine-induced activation of inducible nitric oxide synthase in rat astrocytes

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Received 13 September 2000

Abstract

We investigated the influence of muramyl dipeptide (MDP), a cell wall component of Gram-positive bacteria, on cytokine-induced nitric oxide (NO) production in rat primary astrocytes. MDP alone did not induce NO release in astrocyte cultures. However, MDP increased astrocyte NO production and subsequent nitrite accumulation triggered by IFN-γ. IFN-γ-activated expression of mRNA for inducible NO synthase (iNOS) and iNOS transcription factor interferon regulatory factor-1 (IRF-1) was markedly enhanced in astrocytes treated with MDP. The potentiating effect of MDP on IFN-γ-induced NO production in astrocytes was completely blocked with protein tyrosine kinase (PTK) inhibitor genistein or mitogen activated protein kinase (MAPK) inhibitor PD98059. In contrast, protein kinase C (PKC) inhibitor calphostin C did not affect ability of MDP to augment IFN-γ-triggered astrocyte NO synthesis. These results suggest that MDP synergizes with IFN-γ in the induction of iNOS gene in astrocytes through mechanisms involving PTK and MAPK, but not PKC activation. Finally, MDP also augmented NO production and iNOS mRNA expression in astrocytes treated with IL-1β. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Cellular and molecular biology
Topic: Neuroglia and myelin

Keywords: Muramyl dipeptide; Astrocyte; Nitric oxide; iNOS; Rat

1. Introduction

High levels of pro-inflammatory cytokines and gaseous free radical nitric oxide (NO) are proposed to orchestrate pathophysiologic mechanism(s) associated with bacterial infection of the central nervous system (CNS). The main pathogens causing acute bacterial meningitis belong to both Gram-positive and Gram-negative bacteria such as Escherichia coli and group B streptococci in neonates, Streptococcus pneumoniae and Neisseria meningitidis in children and adults, and intracellular Listeria monocytogenes mainly causing meningitis among newborns, elderly people, and immunocompromised patients [33]. A range of bacterial products and related synthetic compounds have been shown to trigger the transcription of inducible NO synthase (iNOS), the enzyme responsible for generation of high amounts of NO from intracellular L-arginine. Bacterial endotoxin or lipopolysaccharide (LPS) is the major NO-inducing component of Gram-negative bacteria [24], but the components of Gram-positive bacteria that are responsible for iNOS activation are poorly understood. There are reports showing that different Gram-positive bacteria or their cell walls can induce iNOS activity and the production of NO in smooth muscle cells, macrophages or macrophage-cell lines [4,13,23,31]. In the attempts to delineate the molecular substrate responsible for such activity of Gram-positive bacteria, several components were found capable of inducing NO production either in vivo or in vitro: lipoteichoic acid, acting alone [7] or in synergism with peptidoglycan [5,23], superantigens such as streptococcal pyrogenic exotoxins A and C [3], and toxic shock syndrome toxin 1 [44], cell wall serotype.

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polyosides [25], as well as some water soluble protein fractions [40].

It has been shown that several cell types within the central nervous system, including endothelial cells, neurons, microglial cells and astrocytes can be induced to produce NO after stimulation with Gram-positive bacteria [1,21], or their cell wall components [10,19]. Besides being one of the major mechanisms employed by non-specific immune system to combat microorganisms, including gram-positive bacteria [24], high amounts of iNOS-derived NO are toxic for neurons and oligodendrocytes [22]. Thus, NO produced by astrocytes, the predominant cell type in the CNS, in response to bacteria and/or proinflammatory cytokines, may play an important role in the pathophysiology of bacterial meningitis. However, the exact nature of NO-inducing component of Gram-positive bacteria and the requirements for their activation of iNOS in astrocytes have not been delineated.

The aim of the present study was to investigate the role of muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, MDP), the minimal bioactive structure of bacterial cell wall peptidoglycan [6], in the L-arginine-dependent biotransformation pathways leading to formation of NO in rat primary astrocytes.

2. Materials and methods

2.1. Reagents

Fetal calf serum (FCS), RPMI-1640, and phosphate-buffered saline (PBS) were from Flow Laboratories (Irvine, UK). Recombinant rat IFN-γ was obtained from Holland Biotechnology (Leiden, Netherlands). Rat recombinant IL-1β was from Genzyme (Cambridge, MA). N-acetylmuramyl-L-alanyl-D-isoglutamin (MDP) was from GIRPI (Paris, France). Naphthylendiamine dihydrochloride, sulfanilamide, genestein (GEN), calphostin (CAL), polymyxin B sulfate, and L-leucine-methyl-ester (L-LME) were from Sigma (St. Louis, MO). PD98059 was from Research Biochemicals International (Natic, MA). Moloney leukemia virus reverse transcriptase and random primers. PCR amplification of cDNA with primers specific for iNOS/IRF-1 and GAPDH as a house-keeping gene, was carried out in the same tube in a Thermojet (Eurogentec, Seraing, Belgium) thermal cycler as follows: 30 s of denaturation at 95°C, 30 s of annealing at 53°C, and 30 s of extension at 72°C. Number of cycles (30 for both iNOS and IRF-1, and 25 for GAPDH) ensuring non-saturating PCR conditions was established in preliminary experiments. For iNOS, the sense primer was 5'-AGAGAGATCCGGTTCACA-3', and the antisense primer was 5'-CACGAACTGAG-GGTACA-3' corresponding to positions 88–105 and 446–500, respectively, of the published rat iNOS mRNA sequence (GenBank accession number S71597); the PCR product was 376 bp long. For IRF-1, the primers were: sense, 5'-GACGAGAGAGGAAACAG-3'; antisense, 5'-TAACCTCCCTCCCTCATCC-3', corresponding to positions 483–500 and 881–899, respectively, of the published rat IRF-1 mRNA sequence (M34253); the PCR product dislodge microglia and oligodendrocytes and astrocytes were further purified by repetition of trypsinization (0.25% trypsin and 0.02% ethylene diamine tetra-acetic acid) and replating. The cells used in experiments described here were obtained after third to fourth passage when they were more than 97% positive for glial fibrillary acidic protein, an astrocyte-specific intermediate filament component, and less than 1% positive for microglial surface molecule CD11b. For nitrite measurement, after conventional trypsinization procedure, astrocytes (8×10^6/well) were seeded in flat bottomed 96-plates (Flow) in 200 μl medium in the presence of cytokines, MDP, and inhibitors of intracellular signaling pathways (GEN, CAL or PD98059), as indicated in Results. For RNA isolation, astrocytes (5×10^6/well) were incubated in 24-well plates (Flow), in the presence of cytokines and MDP.

2.3. Nitrite measurement

Nitrite accumulation, an indicator of NO production, was measured in 72 h culture supernatants using the Griess reagent [17]. Briefly, 50 μl aliquots of culture supernatants were mixed with an equal volume of Griess reagent (a mixture at 1:1 of 0.1% naphthylenediamine dihydrochloride and 1% sulfanilamide in 5% H₃PO₄) and incubated at room temperature for 10 min. The absorbance at 570 nm was measured in an automated Titertek Multiscan reader (Flow). The nitrite concentration was calculated from a NaNO₂ standard curve.

2.4. Determination of iNOS and IRF-1 mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

After 6 h of incubation total RNA was isolated with RNA Isolator, according to manufacturer’s instruction. RNA was reverse transcribed, using Moloney leukemia virus reverse transcriptase and random primers. PCR amplification of cDNA with primers specific for iNOS/IRF-1 and GAPDH as a house-keeping gene, was carried out in the same tube in a Thermojet (Eurogentec, Seraing, Belgium) thermal cycler as follows: 30 s of denaturation at 95°C, 30 s of annealing at 53°C, and 30 s of extension at 72°C. Number of cycles (30 for both iNOS and IRF-1, and 25 for GAPDH) ensuring non-saturating PCR conditions was established in preliminary experiments. For iNOS, the sense primer was 5'-AGAGAGATCCGGTTCACA-3', and the antisense primer was 5'-CACGAACTGAG-GGTACA-3' corresponding to positions 88–105 and 446–463, respectively, of the published rat iNOS mRNA sequence (GenBank accession number S71597); the PCR product was 376 bp long. For IRF-1, the primers were: sense, 5'-GACGAGAGAGGAAACAG-3'; antisense, 5'-TAACCTCCCTCCCTCATCC-3', corresponding to positions 483–500 and 881–899, respectively, of the published rat IRF-1 mRNA sequence (M34253); the PCR product...
was 417 bp long. The primers for GAPDH were: sense, 5′-GAAGGTGTCAGCCAAAAGC-3′; antisense, 5′-GGA-TGCAGGATGATGTTCT-3′, corresponding to positions 371–388 and 646–665 of the published rat GAPDH mRNA sequence (AB017801); the PCR product was 295 bp long. PCR products were visualized by electrophoresis through agarose or polyacrylamide gel stained with ethidium bromide. Gels were photographed and results were analyzed using NIH Image 1.61 PPC software. Relative expression of iNOS and IRF-1 mRNA was calculated as a ratio between densities of the iNOS/IRF-1 and GAPDH bands.

2.5. Statistical analysis

To analyze the significance of the differences between various treatments performed in triplicates, we used analysis of variance (ANOVA), followed by Student–Newman–Keul’s test. A P-value less than 0.05 was considered significant.

3. Results

3.1. MDP enhances NO production in IFN-γ-stimulated rat primary astrocytes

Non-stimulated astrocytes did not produce detectable levels of nitrites, and MDP by itself, in a wide range of concentrations (1–100 µg/ml), did not have any stimulatory effects on NO production in astrocytes. Proinflammatory cytokine IFN-γ (1000 U/ml) induced by itself significant up-regulation of NO production, measured as a rise of nitrite concentrations in astrocyte cultures (Fig. 1), in accordance with previous results showing iNOS expression and NO production in cytokine-stimulated rodent primary astrocytes [11,15]. Protein synthesis inhibitor cycloheximide (1 µg/ml), as well as aminoguanidine (1 mM), a NOS inhibitor which preferentially inhibits its inducible isoform [27], both completely prevented IFN-γ-induced nitrite accumulation (not shown), indicating that NO in astrocytes was generated via iNOS-dependent l-arginine-NO pathway. We next examined nitrite levels in astrocyte culture supernatants after stimulation with IFN-γ in combination with MDP (1, 10 and 100 µg/ml), and the results revealed statistically significant dose-dependent rise in NO production. The potentiation of NO production was similar at 10 and 100 µg/ml of MDP and ranged between twofold to fivefold in 5 independent experiments (Fig. 1). The observed effect of MDP was not a result of contamination with LPS, since MDP+iNOS-γ-induced NO production (19.7±2.5 µM) was not affected by LPS-inactivating substance polymyxin B (20 µg/ml) (20.9±3.4 µM, P>0.05). In contrast, while IFN-γ-triggered NO synthesis (8.9±1.4 µM) in astrocytes was enhanced by 2 µg/ml LPS (17.4±0.7 µM, P<0.05), polymyxin B completely abolished this LPS effect (10.2±0.2 µM; P<0.05 in comparison with IFN-γ+LPS-treatment). In addition, similar potentiation of NO release in IFN-γ-treated astrocyte cultures was obtained with MDP purchased from another supplier (Sigma; St. Louis, MO). Finally, we excluded the influence of residual microglial contamination by using lysosomotropic agent LLME, which is selectively toxic for macrophages and microglia [16]. While peritoneal macrophages were all killed upon 60 min exposure to 10 mM LLME (not shown), 60 min preincubation with LLME did not affect IFN-γ-induced nitrite accumulation in astrocyte cultures (4.1±0.5 µM vs. 4.1±0.8 µM for control and LLME-treated cells, respectively; P>0.05), indicating that microglia did not contribute to NO generation in our experimental system. Similarly, LLME did not alter MDP-mediated augmentation of NO release in IFN-γ-treated astrocytes (14.7±0.6 µM vs. 16.4±1.2 µM for control and LLME-treated cells, respectively; P>0.05).

3.2. MDP stimulates IFN-γ-induced expression of iNOS and IRF-1 mRNA in astrocytes

To determine whether MDP affects steady state iNOS mRNA levels in IFN-γ treated astrocytes, RT-PCR for iNOS mRNA was performed. Unstimulated astrocytes did not express iNOS mRNA. As expected, IFN-γ caused significant accumulation of iNOS mRNA in astrocyte cultures (Fig. 2). While MDP alone did not induce iNOS message, the addition of MDP further elevated the expres-
Fig. 2. MDP stimulates IFN-γ-induced expression of iNOS and IRF-1 mRNA in astrocytes. Astrocytes (5×10⁵/well) were incubated with or without IFN-γ (1000 U/ml), in the presence of absence of MDP (10 µg/ml). After 6 h, RNA was isolated and the expression of IRF-1 and iNOS mRNA was assessed by RT-PCR. Results from the representative of three separate experiments are presented as relative expression of iNOS/IRF-1 mRNA in comparison to GAPDH.

3.3. Signaling pathways involved in IFN-γ+MDP-induced NO production in astrocytes

To gain some insight into intracellular signals responsible for IFN-γ+MDP-mediated NO production, we used inhibitors of protein tyrosine kinase (PTK), protein kinase C (PKC), and mitogen activated protein kinase (MAPK) pathway—GEN, CAL and PD98059, respectively. Very low basal NO production was not affected by any of the agents tested (data not shown). Each of the three inhibitors almost completely blocked NO synthesis in IFN-γ-treated astrocyte cultures (Fig. 3), without reducing cell viability (not shown), indicating that activation of PTK, PKC, and MAPK is necessary for IFN-γ induction of astrocyte iNOS. MDP-mediated enhancement of IFN-γ-induced NO synthesis was abolished in the presence of GEN (20 µM) or PD98059 (40 µM) (Fig. 3). In contrast, although suppression of IFN-γ-activated NO production was comparable in astrocytes treated with GEN, PD98059, or CAL...
inhibition of PKC activity with CAL did not significantly affect NO synthesis induced by combination of IFN-γ and MDP (Fig. 3). These results suggest that cooperation between IFN-γ and MDP in the iNOS induction in astrocytes involves PTK and MAPK, but not PKC activation.

3.4. MDP enhances NO production and iNOS mRNA expression in IL-1-treated astrocytes

Finally, in order to find out whether the potentiating effect of MDP on NO production was selectively expressed only when astrocytes were exposed to IFN-γ, we analyzed the NO production and iNOS mRNA expression in astrocytes treated with IL-1 in the presence of MDP (10 μg/ml). Exposure of rat astrocytes to IL-1β (10 ng/ml) alone caused an accumulation of nitrite in culture supernatants and induced expression of iNOS mRNA (Fig. 4) in cultivated cells. Similarly to the effects observed in IFN-γ-stimulated astrocytes, MDP enhanced IL-1-induced NO production and further elevated the expression of iNOS mRNA in IL-1-treated astrocytes (Fig. 4).

4. Discussion

In this study we showed that muramyl dipeptide, the minimal bioactive structure of bacterial cell wall peptidoglycan potentiates cytokine-induced iNOS mRNA expression and NO production by rat astrocytes. There are numerous data showing that MDP can induce NO production in macrophages either alone [29,32], or in the combination with various proinflammatory cytokines [34,35]. However, so far the only known effects of MDP on astrocytes are those showing that MDP induces IFN-γ-independent MHC class II expression and production of prostaglandine D2 in rat astrocyte cultures [26,43]. Therefore, the potentiation of cytokine (IL-1, IFN-γ)-induced NO production in astrocytes by MDP, to the best of our knowledge, is the first report of that kind.

Potentiation of astrocyte NO release by MDP was accompanied by enhanced expression of iNOS mRNA, confirming that MDP affected the induction phase of iNOS activation. Previous results indicate that different signaling pathways involving PTK, PKC, and MAPK activation, are involved in the induction of astrocyte iNOS [2,9,14]. Indeed, interference with either PTK, PKC, or MAPK activity, almost completely prevented IFN-γ-triggered astrocyte NO release in our study. This is consistent with findings that tyrosine phosphorylation of STAT1, leading to activation of iNOS transcription factor IRF-1, is necessary for IFN-γ induction of iNOS in rat glial cells [20]. Recently, activation of MAP kinase pathway also has been linked to the IRF-1 induction [8], while it has been well documented that iNOS activation in IFN-γ-stimulated macrophages depends on translocation of PKC from cytosol to cell membrane [37]. However, our data suggest that synergistic cooperation between IFN-γ and MDP in astrocytes, while being completely dependent on PTK or MAPK activity, probably does not involve activation of PKC. Since NO synthesis in astrocytes treated with IFN-γ alone was abolished by PKC inhibition, it seems that MDP provides signal(s) that can replace iNOS-inducing activity of PKC. This is concordant with observation that PKC activation might lead to, but is not absolutely required for iNOS induction in astrocytes [38].

The downstream events responsible for the enhancement of iNOS induction by MDP might involve activation of IRF-1, as indicated by further increase of IFN-γ-triggered accumulation of this iNOS transcription factor in MDP-treated astrocytes. LPS, a cell wall component of gram-negative bacteria and a well known activator of iNOS [24], also has the ability to augment IFN-γ-induced activation of IRF-1 [8]. Accordingly, our preliminary results with simultaneous MDP/LPS treatment indicate that MDP and LPS might share the receptor and/or signaling pathways.
involved in iNOS activation in astrocytes (unpublished observation). This is consistent with previously published ability of both agents to bind to monocyte CD14 [42], a molecule also involved in LPS induction of iNOS in glial cells [12]. However, LPS activation of another iNOS transcription factor, NF-κB, has been regarded as a major mechanism behind synergistic action of IFN-γ/LPS combination in the induction of macrophage iNOS [24]. Since MDP can induce NF-κB activity in various cell types [39], it is possible that activation of this transcription factor in astrocytes might be responsible for LPS-like iNOS-inducing action of MDP in these cells. While our data are consistent with transcriptional regulation of iNOS by MDP, we can not exclude the possibility that MDP could have affected stability of iNOS mRNA. Interestingly, it has been proposed that PKC activity is involved in stabilization of IFN-γ-induced iNOS message in macrophages [18]. Thus, the assumption that MDP might prevent degradation of iNOS mRNA in astrocytes is actually concordant with our observation that MDP stimulates astrocyte NO production by providing a PKC-replacing signal.

Finally, we have shown that MDP-mediated enhancement of iNOS induction in astrocytes was not specific for IFN-γ stimulation, as it was readily observed also in IL-1-treated cells. Elevated levels of IL-1 were found in cerebrospinal fluid taken from both patients [30] and animals with bacterial meningitis in the early stage of infection [41], and antibodies to IL-1 prevented inflammation in rabbits after intracisternal challenge with pneumococci [36]. Therefore, the synergistic induction of iNOS in astrocytes by MDP and IL-1, produced intracerebally by blood derived neutrophils and macrophages, and resident cells such as astrocytes and microglia, might be particularly important in the early phases of infection, during which the IFN-γ-producing T cells are absent from the CNS.

Therefore, our observations suggest that MDP-mediated induction of astrocyte iNOS may be an important determinant of the CNS inflammatory response to Gram-positive infection, contributing to both bacterial clearance and inflammatory destruction of self tissues.

Acknowledgements

This work was supported by grants from Ministry of Science and Technology, Republic of Serbia, Yugoslavia.

References


