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Original article

Inhibition of inflammatory mediator secretion by (−)-DHMEQ in mouse bone marrow-derived macrophages

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Abstract

Previously, we designed and synthesized a new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), and found that racemic DHMEQ inhibited cytokine secretion and phagocytosis by cells of the macrophage cell line RAW264.7. In the present research, we looked into the effect of optically active (−)-DHMEQ on the NO production, inflammatory cytokine secretion, and prostaglandin secretion in mouse bone marrow-derived macrophages (BMMs). We also studied the effect of (−)-DHMEQ on the differentiation of macrophages. DHMEQ inhibited lipopolysaccharide (LPS)-induced NF-κB activation. It also inhibited the expression of inducible NO synthase (iNOS) and NO production induced by LPS. Using enzyme-linked immunosorbent assays, we showed DHMEQ to inhibit LPS-induced secretion of IL-6 and TNF-α. It also inhibited COX-2 expression and prostaglandin E_2 production and secretion. It did not inhibit the phagocytosis of fluorescently labeled *Escherichia coli* by BMMs treated with LPS, unlike in the case of RAW264.7 cells. Next we examined the effect of the inhibitor on M-CSF-induced differentiation of bone marrow cells to macrophages. DHMEQ showed no effect on the differentiation in terms of reactive oxygen species production and F4/80 expression. However, although BMM incorporated oxidized LDL to give rise to foam cells, the (−)-DHMEQ-treated bone marrow cells did not take up oxidized LDL. Taken together, our data show that (−)-DHMEQ inhibited LPS-induced activation of BMM in terms of NO and cytokine secretion, but its effect on phagocytosis differed between BMMs and RAW264.7 cells. We also found that the functional differentiation into macrophages was inhibited by (−)-DHMEQ.
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Keywords: NF-κB; (−)-DHMEQ; Bone marrow-derived macrophage; Interleukin; Inducible nitric oxide synthase; Foam cell

1. Introduction

Macrophages play an important role in immune reactions, allergy, and inflammation [1]. Excess macrophage activation may also enhance solid tumors, diabetes mellitus, and neural disorders such as Alzheimer's and Parkinson's diseases. Therefore, inhibition of excess macrophage activities should be useful as chemotherapy for these diseases. For cell culture studies, the mouse cell line RAW264.7 is often used as a model of macrophages. However, this cell line is tumorigenic; and, therefore, bone marrow-derived macrophages (BMMs) in primary cultures should better reflect the macrophages of patients. Mouse bone marrow cells can be caused to differentiate into macrophages by treatment with M-CSF.

In response to microbes and their products such as lipopolysaccharide (LPS), macrophages secrete various inflammatory cytokines including interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor (TNF)-α through the activation of nuclear factor (NF)-κB, as well as express NF-κB-dependent inducible NO synthase (iNOS [2,3]) and cyclooxygenase-2 (COX-2 [4]). NF-κB is activated by extracellular signals mainly through the various Toll-like receptors in macrophages. Detection and response to microbial infections by the immune system depend on a family of pattern recognition receptors called Toll-like receptors (TLRs [5]). These receptors have been evolutionally conserved to recognize pathogen-associated molecular patterns (PAMPs), including molecules from Gram-positive and -negative bacteria, DNA

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and RNA viruses, fungi, and protozoa; and they show considerable target specificity [6]. TLR2 and TLR4 are the principal receptors for the recognition of various bacterial cell wall components. TLR4 is crucial for the effective host cell responses to LPS of Gram-negative bacteria [7]. Delivery of LPS to TLR4 requires the accessory proteins LBP (LPS-binding protein found in serum), CD14, and MD-2 (the latter two proteins either exist in soluble form or are bound to the cell membrane or to TLR itself) [8]. There are additional cell-surface molecules, such as integrins CD11b/CD18, that may facilitate cellular responses to LPS [9]. TLR4 is also involved in the host responses to pneumolysin, a major virulence factor of *Streptococcus pneumoniae*, and proteins derived from respiratory syncytial virus [10,11]. Arditi's group demonstrated that TLR-4 is preferentially expressed by macrophages in murine and human lipid-rich atherosclerotic lesions, suggesting that TLR-4 may provide a potential pathophysiological link between infection and atherosclerosis [12].

TLR-4 activates TAK1, NIK, and IKK, sequentially, to induce phosphorylation and degradation of IκB-α [13–15].

In the course of our search for inhibitors of NF-κB, we designed and synthesized a new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ, Fig. 1 [16]). DHMEQ was found to inhibit nuclear translocation of NF-κB in TNF-α-treated Jurkat and COS-1 cells [17] as well as LPS-induced cytokine secretion and phagocytosis in mouse macrophage cell line RAW264.7 [18]. DHMEQ used to be prepared as its racemic form; however, for the development of it as a chemotherapeutic agent, the optically active DHMEQ was prepared [19] (−)-DHMEQ is several times more active than (+)-DHMEQ in inhibiting NF-κB. In the present study, we employed (−)-DHMEQ to inhibit NF-κB in macrophages. We looked into the effect of (−)-DHMEQ on NO production, cytokine secretion, COX-2 expression, and phagocytic activity by BMM.

2. Materials and methods

2.1. Reagents

(−)-DHMEQ was synthesized in our laboratory as previously described [19]. Lipopolysaccharide (LPS; #L2654), low-density lipoprotein (LDL) from human plasma (#L-5402) and Sudan Black B were purchased from Sigma Chemical (St Louis, MO), and recombinant human macrophage colony-stimulating factor (M-CSF) from R&D Systems (Minneapolis, MN). Anti-iNOS antibody came from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-COX-2 antibody from BD Biosciences (San Jose, CA).

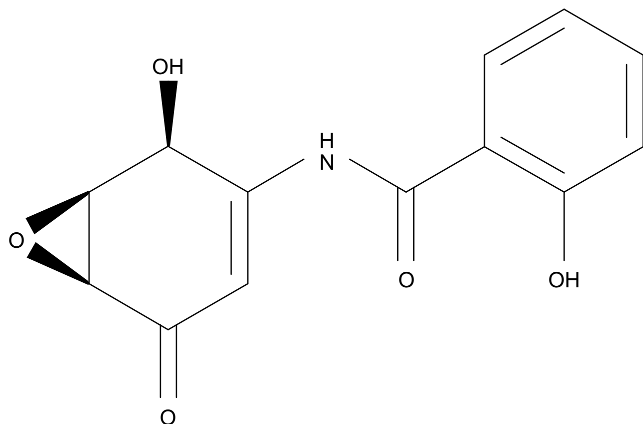

Fig. 1. Structure of (−)-DHMEQ.

2.2. Cell culture

Preparation of bone marrow-derived monocyte/macrophage precursor cells (BMMs) was carried out as described previously [20,21]. In brief, non-adherent bone marrow cells derived from 8-week-old ICR mice (Charles River Japan, Tokyo, Japan) were seeded at 5×10^5 cells/well into 24-well plates or at 5×10^6 cells/dish into 60-mm dishes and cultured in α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (JRH Bioscience, Lenexa, KS, USA), 200 μg/ml kanamycin, 100 units/ml penicillin G, and 2.25 g/l $NaHCO_3$ containing 10 ng/ml M-CSF. After 2 days, adherent cells were used as BMMs after the nonadherent cells had been washed out of the cultures. These BMMs were further cultured in the presence of 100 ng/ml M-CSF.

2.3. Electrophoresis mobility shift assay (EMSA)

Cells (4×10^5 cells/ml) were seeded in 9 ml of complete medium into 100-mm dishes. On the next day, the volume of the culture medium was adjusted to 6 ml. The cells were then pretreated with DHMEQ for 2 h, after which LPS was added. For the preparation of nuclear extracts, the adherent cells were washed with Ca^{2+}, Mg^{2+}-free PBS (PBS^-) and then scraped into 400 μl of a cold buffer consisting of 10 mM HEPES (pH 7.9), 1.5 mM DTT, and 0.2 mM PMSF. The suspension was subsequently centrifuged at 3500 rpm for 5 min. The resulting supernatant was discarded, and the pellet was resuspended in 40 μl of a buffer consisting of 20 mM HEPES–KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF, and stood for 20 min on ice. The mixture was centrifuged at 15,000 × *g* for 5 min, and the supernatant was recovered as the nuclear extract. The protein content of the extract was determined by using Coomassie Protein Assay Reagent (Pierce Endogen, Rockford, IL). For the preparation of radioactive probes a double-stranded oligonucleotide (GGGGACTTTCC; Promega) at 75 pmol/ml and containing the NF-κB consensus sequence was end-labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase (Takara, Ohtsu, Japan) in 2 ml of a buffer consisting of 500 mM Tris–HCl (pH 8.0), 100 mM $MgCl_2$, and 50 mM DTT. Then, 4 μl of 5 × binding buffer (75 mM Tris–HCl, pH 7.0, containing 375 mM NaCl, 7.5 mM EDTA, 7.5 mM DTT, 37.5% glycerol, 1.5% NP-40, and 1.25 mg/ml BSA) and 1 μl poly dI-dC (1 μg/ml) was added to 5 μg of the nuclear extract, and the whole volume was adjusted to 12 μl with water. After the addition of 3 μl of the DNA probe, the mixture was incubated at 25 °C for 20 min. Each extract was electrophoresed

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