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RAPID COMMUNICATION

κ-Opioid Modulation of Human Microglial Cell Superoxide Anion Generation

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ABSTRACT. Opioids have been postulated to play an immunomodulatory role in the CNS. Recently, we found that priming microglia with interferon (IFN)-γ or tumor necrosis factor (TNF)-α resulted in an enhanced production of superoxide anion, a reactive oxygen intermediate that may be pathogenic during brain inflammation. In the present study, we investigated the effects of *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (U50,488), a selective κ-opioid ligand, on microglial cell superoxide production when cells were primed with cytokines or stimulated with phorbol myristate acetate. While treatment of microglial cells with U50,488 had little effect on nonstimulated or stimulated superoxide production, this opioid inhibited (by >70%) the priming effects of cytokines. Maximal inhibition of microglial cell superoxide generation by U50,488 was observed at 10 nM for the priming effect of interferon-γ and at 1 μM for tumor necrosis factor-α. Pretreatment of microglial cell cultures for 30 min with an equal concentration of the selective κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI) completely blocked the inhibitory effect of U50,488. The results of this study suggest that κ-opioids may have therapeutic potential in inflammatory diseases of the CNS involving reactive oxygen intermediates produced by activated microglia. BIOCHEM PHARMACOL **56**;3:285–288, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. interferon; tumor necrosis factor; reactive oxygen intermediate; inflammation; brain

Endogenous opioid peptides have been found to modulate several CNS functions via G-protein-coupled opioid receptors [1–3]. Considerable evidence indicating that opioids affect immunologic responses within the CNS also exists [4]. Activation of opioid receptors with selective ligands, for example, has been shown to alter the phagocytic [5] and chemotactic [6] activities of microglia, the resident macrophages of the brain. Microglia have been implicated in the pathogenesis of CNS infections and neuronal injury [7]. The mechanisms underlying microglia-mediated neurodegeneration are unclear; however, the release of mediators by these mononuclear phagocytes has been proposed to play a role [8]. Among these mediators, ROIs†, i.e. superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite, are thought to contribute to pathologic processes accompanying CNS inflammation [9]. For example, microglial cell ROI production has been reported to cause neuronal cell death [10]. Human microglia constitutively express KORs, and *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (U50,488) has been found to modulate HIV-1 expression in acutely infected microglia [11]. KOR ligands also have been reported to up-regulate cytokine expression in human fetal glial/neuronal cell cultures [12]. Given these immunomodulatory properties of κ-opioids [4], in the present study we tested the hypothesis that U50,488, a selective KOR ligand, would modulate microglial cell superoxide production.

MATERIALS AND METHODS

Reagents

The selective KOR ligand U50,488 was a gift of the Pharmacia & Upjohn Co., and the κ-selective antagonist nor-BNI was provided by P. S. Portoghese (University of Minnesota). Recombinant human TNF-α and IFN-γ were obtained from R&D Systems, Inc. PMA, and other culture reagents were purchased from the Sigma Chemical Co. Fetal bovine serum was obtained from HyClone Laboratories. Anti-CD68 antibodies (a microglial cell marker) were obtained from DAKO.

Microglial Cell Cultures

Human fetal brain tissue was obtained from 18- to 22-week-old aborted fetuses under a protocol approved by the Human Subjects Research Committee at our institution, as previously described [8]. Briefly, brain tissues were dissoci-

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† *Abbreviations:* IFN, interferon; KOR, κ-opioid receptor; nor-BNI, nor-binaltorphimine; PMA, phorbol myristate acetate; ROI, reactive oxygen intermediate; and TNF, tumor necrosis factor.

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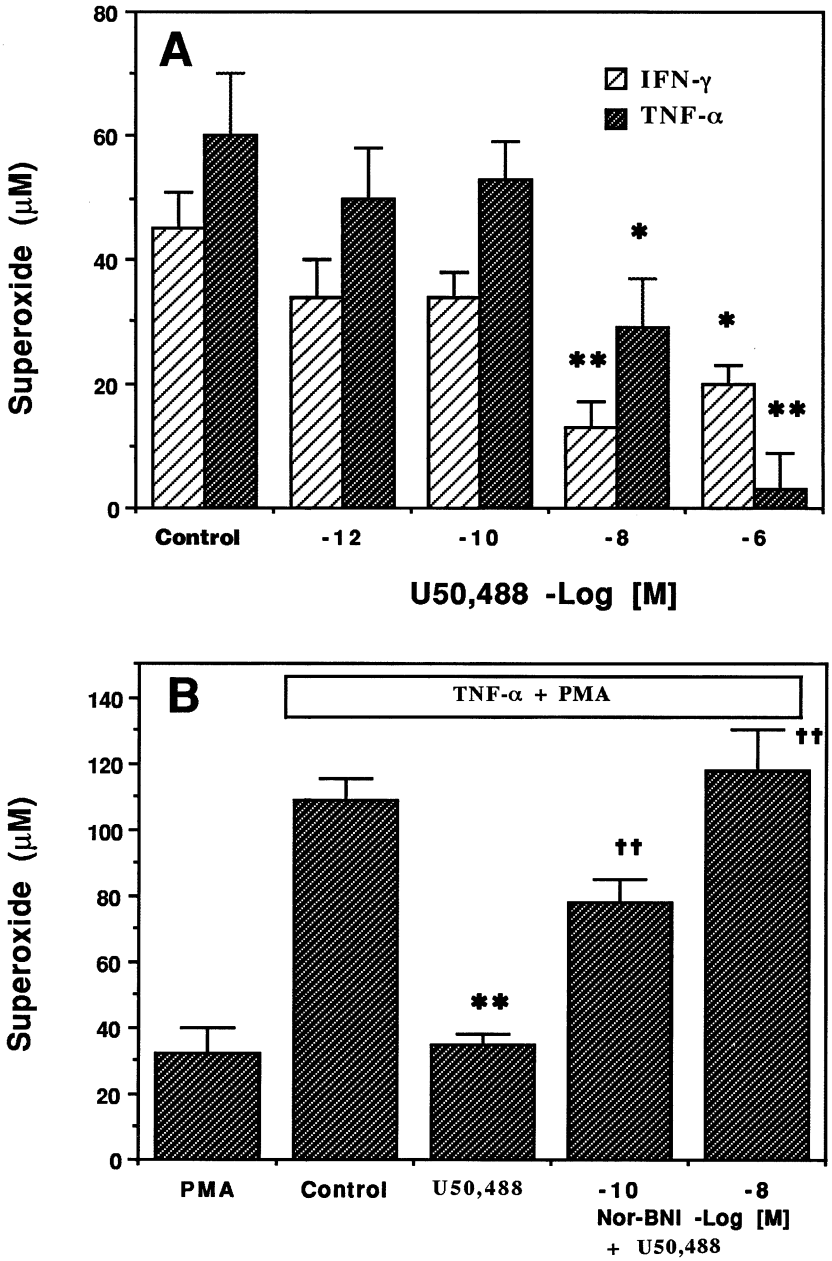

FIG. 1. U50,488 inhibition of superoxide generation by cytokine-primed microglia. (A) Microglial cell cultures were pretreated with the indicated concentrations of U50,488 for 24 hr prior to exposure to 200 U/mL of IFN-γ or 10 ng/mL of TNF-α for an additional 6 hr followed by stimulation with 50 nM PMA for 90 min. (B) Microglial cell cultures were incubated in medium without (control) or with 10 nM U50,488 or in the presence of indicated concentrations of nor-BNI for 24 hr before exposure to TNF-α for 6 hr. Data are means (± SEM) of quadruplicates and are representative of three separate experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs corresponding nontreated control cultures; and (††) $P < 0.01$ vs U50,488 alone.

ated following a 30-min trypsinization (0.25%) and were plated in 75-cm^2 Falcon culture flasks in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). The medium was replenished 1 and 4 days after plating. On day 12 of culture, plates were shaken gently, and harvested microglial cells were plated onto wells of 96-well plates at a density of 2×10^4/well. Purified microglia were composed of a cell population of which >99% was stained with anti-CD68 antibodies.

Superoxide Production

The production of superoxide by glial cells was quantified by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*, as previously described [8]. For studying the effects of U50,488 on the priming of microglia by cytokines, microglial cell cultures were incubated first with U50,488 for 24 hr followed by the addition of either 200 units/mL of IFN-γ or 10 ng/mL of TNF-α for 6 hr. Cell cultures were washed and then stimulated with 50 nM PMA. After a 90-min incubation in a 5% CO_2 incubator at 37°, plates were read at 550 nm. For comparison of multiple means, analysis of variance was used, followed by *post hoc* analysis with Scheffe's F-test.

RESULTS AND DISCUSSION

Treatment of microglial cell cultures with U50,488 alone at concentrations between 1 pM and 1 μM did not trigger any

detectable superoxide anion (data not shown), suggesting the lack of a direct stimulatory effect of U50,488 on microglial cell respiratory burst activity. Previously, we demonstrated that human fetal microglial cells generate increased amounts of superoxide upon stimulation with 50 nM PMA [8]. Pretreatment of microglial cell cultures with U50,488 at concentrations between 1 fM and 1 μM did not affect PMA-triggered superoxide production (data not shown). These findings are consistent with the notion that ligand activation of KOR does not have a direct effect on PMA-stimulated microglial cell respiratory burst activity.

Two proinflammatory cytokines (IFN-γ and TNF-α) have been reported to prime microglial cells for enhanced superoxide production [9]. IFN-γ is derived from activated T lymphocytes, whereas TNF-α is produced principally by activated mononuclear phagocytes, including microglia in the CNS. Neither of these cytokines alone alters superoxide production; however, both cytokines are capable of priming microglia for enhanced generation of superoxide upon PMA stimulation [8]. Thus, we next evaluated the effect of U50,488 on the priming of microglia by these cytokines. Figure 1A shows that treatment of microglial cell cultures with U50,488 for 24 hr interfered in a concentration-dependent manner with the priming effects of IFN-γ and TNF-α on superoxide production. Maximal inhibition (approximately 70 and 95% for the priming effect of IFN-γ and TNF-α, respectively) of microglial cell superoxide generation by U50,488 was observed at 10 nM and 1 μM U50,488, respectively. The IC_{50} for U50,488-induced suppression of the TNF-α priming effect was approximately 10 nM. Pretreatment of microglial cell cultures for 30 min with equimolar concentrations of the KOR antagonist nor-BNI completely blocked the inhibitory effect of U50,488, whereas 100-fold lower concentrations resulted in a partial effect (i.e. 58% inhibition) (Fig. 1B). These findings suggest that the suppressive activity of U50,488 is mediated through a mechanism involving KOR. To evaluate whether the inhibitory effect of U50,488 could be found with other incubation times, we incubated microglial cells with 10 nM U50,488 for various time periods prior to exposure to 20 ng/mL of TNF-α. The inhibitory effects of U50,488 were 15, 86, 96, and 48% when cells were pretreated for 48, 24, 6, and 0 hr prior to a 6-hr exposure to TNF-α and stimulation with PMA. These findings suggest that the time of cell exposure to opioids is critical for maximal inhibition of microglial cell respiratory burst activity.

PMA is known to stimulate the microglial cell respiratory burst via a protein kinase C signal transduction pathway [8]. Because U50,488 did not alter PMA-triggered microglial cell superoxide production, it is unlikely that U50,488 interferes with this signaling pathway. Although the intracellular signaling pathway of the priming effects of IFN-γ and TNF-α on superoxide production is unknown, it has been suggested that potentiation of protein kinase C activity may account for their priming effects [8]. Thus, the inhibitory activity of U50,488 on cytokine priming of microglia may occur via interaction with the seven-transmembrane G-protein-coupled KOR linked to protein kinase C.

In summary, this is the first report documenting that activation of KOR on microglia has an immunomodulatory effect on the production of ROI by cytokine-primed microglial cells in culture. It has long been known that microglia migrate to, proliferate, and differentiate at sites of inflammation [13] and may play a neurodestructive role [7]. U50,488 has been found previously to be neuroprotective in several animal models [14–16]. The findings in the present study suggest that the capacity of microglia to produce ROI at such inflammatory sites is influenced by κ-opioids within the local milieu. Although the biological importance of the findings in this *in vitro* study is unknown, it is possible that the immunomodulatory property of κ-opioids may account for, at least in part, their neuroprotective effects. Thus, targeting microglial cell ROI generation by κ-opioids could prove to be of therapeutic benefit in CNS injury, including stroke, infectious diseases, and certain neurodegenerative disorders.

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