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Chloroquine induces the expression of inducible nitric oxide synthase in C6 glioma cells

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Abstract

Chloroquine, a well-known lysosomotropic agent, has long been used for the treatment of malaria and rheumatologic disorders. However, therapeutic doses of chloroquine are known to cause behavioral side effects. In the present study, we investigated whether chloroquine stimulates inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) synthesis in C6 glioma cells. Chloroquine caused dose-dependent increase in iNOS protein expression and NO production in C6 glioma cells. A tyrosine kinase inhibitor (genistein), a protein kinase C (PKC) inhibitor (Ro 31-8220), and a p38 mitogen-activated protein kinase (MAPK) inhibitor (SB 203580) all respectively suppressed chloroquine-induced iNOS expression and NO release from C6 glioma cells. Chloroquine activates p38 MAPK and stimulates PKC-α and -δ translocation from the cytosol to the membrane in C6 glioma cells. Chloroquine-stimulated p38 MAPK activation was blocked by genistein (20 μM), Ro 31-8220 (3 μM), and SB 203580 (10 μM). Incubation of lipopolysaccharide (LPS)-stimulated cells with chloroquine at non-toxic concentrations (10–100 μM) for 48 h increased iNOS expression, and led to a significant loss of adherent cells. Induction of DNA fragmentation in floating cells indicated that the C6 glioma cells were undergoing apoptosis. Taken together, our data suggest that chloroquine may activate tyrosine kinase and/or PKC to induce p38 MAPK activation, which in turn induces iNOS expression and NO production.

Keywords: iNOS; Chloroquine; C6 glioma cells; p38 MAPK

1. Introduction

Chloroquine is an aminoquinoline drug used in the treatment of malaria. Chloroquine is also a beneficial therapeutic agent in systemic lupus, rheumatoid arthritis, and viral infection [1–4]. Chloroquine can pass through the plasma membrane and preferentially concentrates in acidic cytoplasmic vesicles, which increases the cytoplasmic pH. Elevation of the cytosolic pH may influence endocytosis, exocytosis, phagocytosis [5], and other cell functions, such as antigen presentation [6] and iron metabolism [7].

However, administration of therapeutic doses of chloroquine may cause psychosis, delirium, personality changes, and depression [8]. Chloroquine intoxication causes ganglioside storage in nervous tissue [9]. Administration of chloroquine into the brains of young rats induced the formation of lysosome-associated granular aggregates (dense bodies), which closely resembled certain disease states and processes during aging [10]. Chloroquine increases the intracellular level of reactive oxygen species (ROS) and human astrogial

Abbreviations: ATF-2, activating transcription factor 2; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; iNOS, inducible nitric oxide synthase; l-NAC, L-nitro-acetyl-cysteine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/Erk kinase; NF-κB, nuclear factor kappa B; NO, nitric oxide; PC-PLC, phosphatidylcholine-specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species

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cells [11]. On the other hand, chloroquine increased NO synthesis but did not alter NOS expression in endothelial cells [7]. A high level of gene expression of NOS was detected by in situ hybridization in chloroquine-treated mice [12] suggesting that enhancement of NO production may be an important step in chloroquine's action.

NO is a diffusible gas that is generated enzymatically from L-arginine and molecular oxygen by NO synthase. To date, at least three different types of NOSs have been characterized. The endothelial (eNOS) and neuronal (nNOS) types are constitutively expressed; whereas, the inducible type (iNOS) is induced by a variety of signals in many cell lines [13]. Nitric oxide plays an important role in both physiological and pathological conditions. At low concentrations of NO, it has been shown to serve as a neurotransmitter and a vasodilator, while at high concentrations, it is toxic and may be important in several neurodegenerative diseases [14]. Overproduction of nitric oxide in the brain is the biochemical basis of many neuropathological features, of oxidative stress [15], and of neuronal cell death [16,17]. Microglial cell-derived NO can contribute to oligodendrocyte degeneration and neuronal cell death [18,19]. In Alzheimer's disease, neurons are subjected to deleterious cytotoxic effects of activated microglia [20].

In the present study, we present evidence that chloroquine may stimulate iNOS protein expression in C6 glioma cells. We also demonstrate that chloroquine markedly stimulates p38 MAPK activity. Inhibition of p38 MAPK activity and iNOS protein expression by genistein and SB 203580 suggests that chloroquine may activate the pathway of tyrosine kinase to induce p38 MAPK activation. Thus, the protein tyrosine kinase-p38 MAPK pathway may be the upstream signal that contributes to chloroquine-induced iNOS expression. Furthermore, enhancement of LPS-mediated iNOS expression and nitrite production by chloroquine suggests that excessive NO production may contribute to C6 glioma cell apoptosis.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), glutamine, gentamycin, penicillin and streptomycin were purchased from Life Technologies (Gaithersburg, MD). Antibodies specific for iNOS, eNOS, and α-tubulin were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). SB 203580, genistein, and FPT inhibitor-II were purchased from Calbiochem-Novabiochem (San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was purchased from Bio Rad (Hercules, CA). The p38 MAPK activity assay kit was purchased from New England Biolabs (Beverly, MA). Chloroquine and all other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Culture of C6 glioma cells and preparation of cell lysates

C6 glioma cells were cultured in DMEM supplemented with 13.1 mM $NaHCO_3$, 13 mM glucose, 2 mM glutamine, 10% heat-inactivated FCS, and penicillin (100 U ml^{-1})/streptomycin (100 mg ml^{-1}). Cells were attached to a Petri dish after a 24 h incubation. Cells were plated at a concentration of 1×10^5 cells ml^{-1} and used for the experiment when they reached 80% confluency. Cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C. After reaching confluence, cells were treated with various concentrations of chloroquine for indicated time intervals and incubated in a humidified incubator at 37 °C. In some experiments, cells were pre-treated with specific inhibitors as indicated for 30 min before chloroquine treatment. After incubation, cells were lysed by adding lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM DTT, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktails (final concentrations: 0.2 mM PMSF, 0.1% aprotinin, 50 μg ml^{-1} leupeptin). Cells adhering to the plates were scraped off using a rubber policeman and stored at −70 °C for further measurements. For fractionation of cellular extracts for analysis of the translocation of PKC isoforms, cells were homogenized by adding homogenization buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM DTT, and the protease inhibitor cocktails (final concentrations: 0.2 mM PMSF, 0.1% aprotinin, 50 μg ml^{-1} leupeptin). The cell suspension was chilled on ice for 30 min, and then centrifuged at 800 × *g* for 10 min at 4 °C. The supernatant (containing cytosolic and membrane fractions) was then centrifuged at 25,000 × *g* for 15 min at 4 °C. The supernatant represents the cytosolic fraction, and the pellet membrane fraction was resuspended in homogenizing buffer containing 1% NP40. The protein levels of PKC-α and -δ in both fractions were determined by Western blot analysis.

2.3. Polyacrylamide gel electrophoresis and Western blotting

Electrophoresis was ordinarily carried out using different percentages of SDS-polyacrylamide electrophoresis (SDS-PAGE). Following electrophoresis, proteins on the gel were electrotransferred onto a polyvinyldifluoride (PVDF) membrane. After transfer, the PVDF membrane was washed once with PBS and twice with PBS plus 0.1% Tween 20. The PVDF membrane was then blocked with blocking solution containing 3% bovine serum albumin in PBS containing 0.1% Tween 20 for 1 h at room temperature. The PVDF membrane was incubated with a solution containing primary antibodies in the blocking buffer. Finally, the PVDF membrane was incubated with peroxidase-linked anti-mouse IgG antibodies for 1 h and then developed using a LumiGLO chemiluminescence kit (Amersham, UK).