Pharmacological Research 51 (2005) 329–336

Pharmacological research

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

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Accepted 1 October 2004

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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doi:10.1016/j.phrs.2004.10.004

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).

2.4. Measurement of nitrite formation in C6 glioma cultures

C6 glioma cells were cultured in 35-mm Petri dishes. 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, the medium was removed and stored at −80 °C until assaying for nitrite accumulation. Nitrite production was measured by adding 0.15 ml of the cell culture medium to 0.15 ml of Griess reagent [21] in a 96-well plate, which was then incubated in a dark place at 37 °C for 10 min. Absorbance was measured at 540 nm using a microplate reader. A blank was prepared for each experimental condition in the absence of C6 glioma cells, and the absorbance was subtracted from that obtained in the presence of cells.

2.5. Measurement of p38 MAPK activity

p38 MAPK activity was measured using a p38 MAPK activity assay kit (New England Biolabs). Briefly, C6 glioma cells were cultured in 10-cm Petri dishes. 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 washed with phosphate-buffered saline (pH 7.4). Proteins were extracted with lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, and 1 μg ml^{-1} leupeptin). Cell extracts were incubated with anti-phospho-p38 MAPK antibody, which was immobilized to crosslinked agarose hydrazide beads, overnight at 4 °C. The beads were then centrifuged for 30 s at 4 °C. The cell pellet was washed twice with lysis buffer, and then incubated with 50 μl of kinase buffer (25 mM Tris [pH 7.5], 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$) supplemented with 200 μM of ATP and 2 μg of ATF-2 for 60 min at 30 °C. The reaction was terminated by the addition of 3× SDS sample buffer and applied to a 10% SDS-PAGE gel. The phosphorylated ATF-2 was detected using a LumiGLO chemiluminescent reagent, and then exposed to X-ray film.

2.6. Analysis of DNA content and DNA fragmentation in C6 glioma cultures

C6 glioma cells were cultured in 10-cm Petri dishes. After reaching 70% confluence, cells were treated with various concentrations of chloroquine in the absence or presence of lipopolysaccharide for 48 h in a humidified incubator at 37 °C. After incubation, cells were incubated with 50 mg ml^{-1} propidium iodide, and DNA content was measured using a flow cytometry analysis (Becton-Dickinson, San Jose, CA); 15,000 events were analyzed for each sample. In analysis of DNA fragmentation, both the attached and unattached cells were harvested and washed twice with ice-cold PBS, and pellets were lysed at 37 °C in 4 ml of extraction buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA; 20 μg ml^{-1} pancreatic RNase, and 0.5% SDS) for 2 h. After incubation, proteinase K was added at a final concentration of 100 μg ml^{-1}, and the mixture was incubated for another 3 h at 50 °C. The DNA was extracted twice with equal volumes of a phenol and chloroform–isoamyl alcohol mixture (24:1, v:v). The DNA was then precipitated with 0.1 volume of sodium acetate [pH 4.8] and 2.5 volumes of ethanol at −20 °C overnight and centrifuged at 13,000 × *g* for 1 h. Samples were electrophoresed on a 1.5% agarose gel, and the DNA was visualized by ethidium bromide staining.

2.7. Statistical analysis

Results are expressed as the mean ± S.E.M. from the number of independent experiments performed. One-way analysis of variance (ANOVA) and Student's two-tailed *t*-test were used to determine the statistical significance of the difference between means. A *p*-value of less than 0.05 was taken as statistically significant.

3. Results

3.1. Chloroquine stimulated dose- and time-dependent increases in NO release and iNOS induction in C6 glioma cells

Chloroquine stimulated NO production in C6 glioma cells in a dose-dependent manner (Fig. 1A). The EC_{50} of chloroquine-stimulated nitrite accumulation was about 30 μM, with a maximum at 100 μM. Consistent with previous reports [22,23], eNOS was constitutively expressed in C6 glioma cells (Fig. 1B). Chloroquine induced the expression of 130-kDa iNOS but not eNOS in C6 glioma cells (Fig. 1B). The induction of iNOS became apparent at 24 h with a maximum at about 48 h (Fig. 1C). Pre-treatment of C6 glioma cells with L-nitro-acetyl-cysteine (L-NAC), a known glutathione precursor, did not attenuate chloroquine-stimulated nitrite release or iNOS induction suggesting that the chloroquine-induced response is not due to reactive oxygen species (Fig. 2).

3.2. Intracellular signaling pathway of chloroquine-induced iNOS expression and nitrite production in C6 glioma cells

To delineate the roles of protein tyrosine kinase, Ras and MAPK in iNOS induction, we next investigated the effects of a tyrosine kinase inhibitor (genistein), a Ras-farnesyl transferase inhibitor (FPT inhibitor-II), a MEK inhibitor

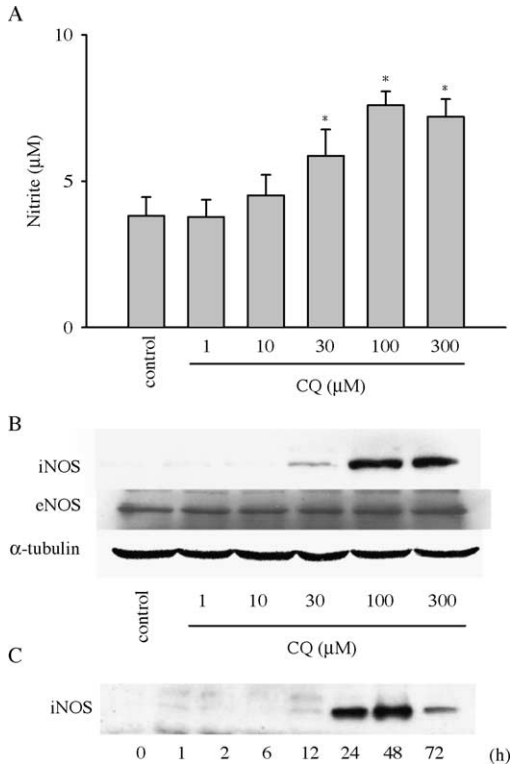

Fig. 1. Dose-dependent increases in nitrite accumulation and iNOS expression caused by chloroquine in C6 glioma cells. Cells were incubated with various concentrations of chloroquine for 48 h, then the medium was removed and analyzed for nitrite accumulation (A). Data represent the mean ± S.E.M. of three independent experiments in triplicate. In (B), cells were incubated with various concentrations of chloroquine for 24 h, and then immunodetected with iNOS- or eNOS-specific antibody as described in Section 2. In (C), cells were incubated with 100 μM chloroquine for various time periods, and then immunodetected with iNOS- or eNOS-specific antibody as described in Section 2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin.

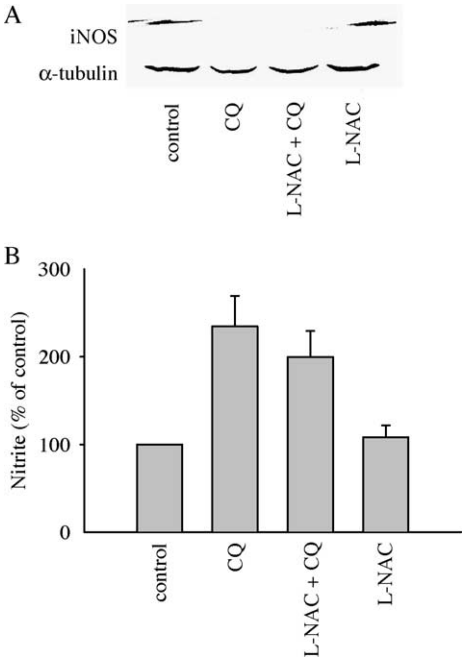

Fig. 2. Effects of L-nitro-acetyl-cysteine (L-NAC) on chloroquine-induced nitrite production in C6 glioma cells. In (A), C6 glioma cells were pre-treated with L-NAC (10 μM) for 30 min before the addition of chloroquine (100 μM) for 24 h. Extracted proteins were then immunodetected with iNOS-specific antibody as described in Section 2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. In (B), cells were pre-treated with L-NAC (10 μM) for 30 min before the addition of chloroquine (100 μM) for 48 h. Then the medium was removed and analyzed for nitrite accumulation. Data represent the mean ± S.E.M. of three independent experiments in triplicate. $^{*}p < 0.05$ as compared with the AGEs-treated group.

(PD 98059), and a p38 MAPK inhibitor (SB 203580) on chloroquine-induced iNOS expression (Fig. 3A). Fig. 3A shows that pre-treatment of cells with genistein (20 μM) or SB 203580 (10 μM) but not the FPT inhibitor II (20 μM) or PD 98059 (10 μM) attenuated chloroquine-stimulated iNOS expression. Furthermore, while the PKC inhibitor (Ro 31-8220, 3 μM) blocked chloroquine-induced iNOS expression, the PKA inhibitor (KT 5720, 3 μM), or the NF-κB inhibitor (PDTC, 25 μM), or the PC-PLC inhibitor (D 609, 50 μM) had no effect on chloroquine-induced iNOS expression in C6 glioma cells (Fig. 3A). Thus, activations of tyrosine kinase, p38 MAPK, and PKC seem to be involved in chloroquine-mediated signal transduction leading to iNOS expression. Chloroquine-stimulated NO production was dose-dependently inhibited by genistein (Fig. 3B), SB 203580 (Fig. 3C), and Ro 31-8220 (Fig. 3D). Previous studies have demonstrated that C6 glioma cells express the PKC-α and -δ isoforms [24]. To confirm that chloroquine does activate PKC in C6 glioma cells, the expression of PKC-α and -δ isoform in cytosol and membrane fractions was examined. In resting cells, PKC-α and -δ were both detected in the cytosolic fraction and in the particulate (membrane) fraction. Treatment of C6 glioma cells with chloroquine (100 μM) resulted in PKC-α and -δ translocation. Increases of PKC in the membrane fraction became evident at 10 min and persisted for 1 h (Fig. 3E). PKC down regulation was observed during a time course of 6 h.

3.3. Activation of p38 MAPK by chloroquine in C6 glioma cells

The data in Fig. 3 suggest that the p38 MAPK-activated pathway might contribute to the signaling mechanism for chloroquine-induced iNOS expression in C6 glioma cells. This notion was supported by the fact that chloroquine activates p38 MAPK in C6 glioma cells. As shown in Fig. 4, addition of chloroquine to C6 glioma cells stimulated an increase in p38 MAPK activity in dose- and time-dependent manners as determined with an immunocomplex kinase assay using ATF-2 as the substrate (Fig. 4). Chloroquine-stimulated ATF-2 phosphorylation was apparent within 5 min, reached a maximum at about 30 min, and then had decreased by 2 h. Western blot analysis using anti-p38 MAPK antibodies indicated that the total protein expression of p38 MAPK was unaffected by treatment of cells with chloroquine. When cells were pre-treated for 30 min with genistein (20 μM), SB 203580

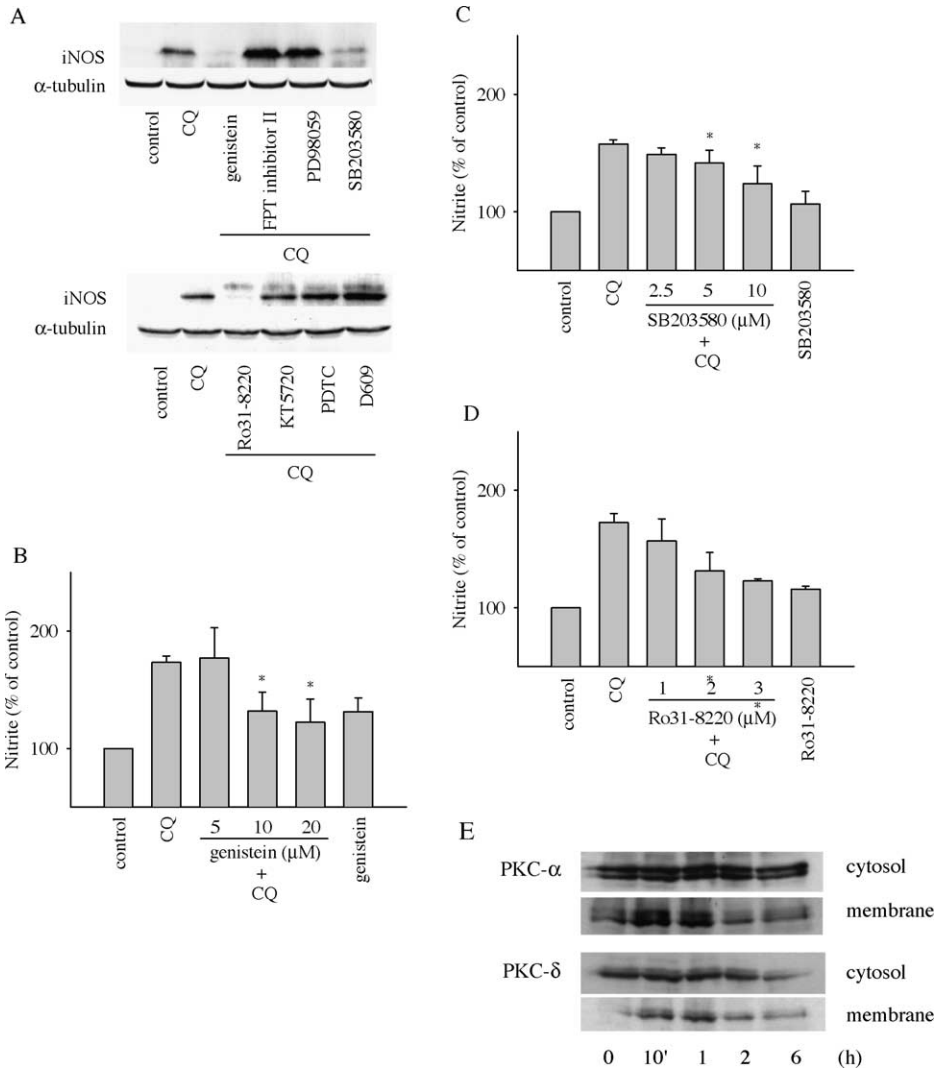

Fig. 3. Effects of genistein, FPT inhibitor-II, PD 98059, and SB 203580 on chloroquine-induced nitrite production and iNOS expression in C6 glioma cells. In (A), cells were pre-treated with genistein (20 μM), FPT inhibitor-II (20 μM), PD 98059 (10 μM), SB 203580 (10 μM), Ro 31-8220 (3 μM), KT 5720 (3 μM), PDTC (25 μM), and D 609 (50 μM) for 30 min before the addition of 100 μM chloroquine for 24 h. Cell lysates were then prepared for immunodetection using an iNOS-specific antibody as described in Section 2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. In (B–D), cells were pre-treated with different concentrations of genistein (5–20 μM), SB 203580 (2.5–10 μM), or Ro 31-8220 (1–3 μM), for 30 min before the addition of 100 μM chloroquine for 48 h. Then, the medium was removed and analyzed for nitrite accumulation. Data represent the mean ± S.E.M. of three independent experiments in triplicate. $^{*}p < 0.05$ as compared with the chloroquine-treated group. In (E), cells were treated with 100 μM chloroquine for the indicated time periods, and then separated into cytosol and membrane factions. Protein in the cytosol and membrane fractions were subjected to Western blot analysis using specific PKC-α and -δ antibodies.

(10 μM), or Ro 31-8220 (3 μM), the chloroquine-induced activation of p38 MAPK was markedly inhibited (Fig. 5). These results suggest that protein tyrosine kinase and PKC act upstream of p38 MAPK.

3.4. Chloroquine enhances LPS-stimulated nitrite production and iNOS protein expression in C6 glioma cells

We evaluated the effect of chloroquine on iNOS expression in the presence of a sub-maximum concentration of LPS (0.5 μg ml^{-1}). As shown in Fig. 6A, LPS (0.5 μg ml^{-1}) stimulated nitrite production in C6 glioma cells, and the response was enhanced by increasing concentrations of chloroquine. Similar enhancement of LPS-induced iNOS induction by chloroquine was observed (Fig. 6B). Chloroquine at 10 μM did not stimulate nitrite production or iNOS expression in C6 glioma cells (Fig. 1). Addition of 10 μM chloroquine enhanced the LPS-stimulated induction of iNOS expression in glioma cells (Fig. 6B). However, incubation of LPS-stimulated cells with chloroquine (100 μM) for 48 h led to a significant loss of adherent cells. The increase in the sub-G1 cell population (Fig. 7A) and induction of DNA fragmentation in floating cells (Fig. 7B) indicates that the C6 glioma cells were undergoing apoptosis. Giving chloroquine at non-toxic concentrations may stimulate excessive NO pro-

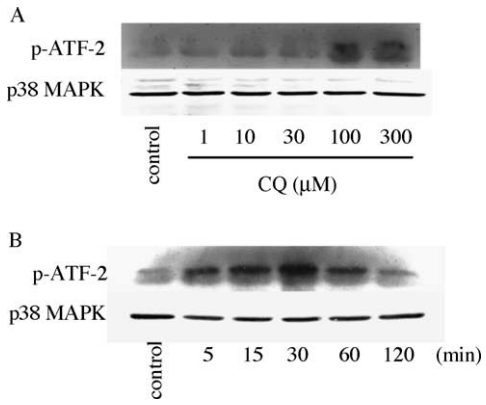

Fig. 4. Chloroquine activation of p38 MAPK in C6 glioma cells. Cells were incubated with various concentrations of chloroquine for 30 min (A), or with 100 μM chloroquine for various time periods (B). Cells were lysed, and the p38 MAPK activity was determined using an immunocomplex kinase assay with ATF-2 as the substrate as described in Section 2. Equal loading in each lane is demonstrated by a similar protein level of p38 MAPK.

duction in activated microglial cells and lead to cell apoptosis.

4. Discussion

In the present study, we demonstrate that chloroquine increases nitrite production and iNOS expression in C6 glioma cells. We elucidate the intracellular signal transduction mechanisms by which chloroquine stimulates iNOS expression in C6 glioma cells. We found that protein tyrosine kinase, p38 MAPK, and PKC are involved in chloroquine-stimulated iNOS protein expression, while NF-κB, cAMP-dependent protein kinase, and p44/42 MAPK are not involved in the signaling pathway. We present evidence that chloroquine stimulates p38 MAPK activity, and that this effect can be inhibited by genistein and Ro 31-8220, suggesting that protein tyrosine kinase and PKC are the upstream regulators of p38 MAPK. Chloroquine greatly enhances iNOS expression in LPS-treated C6 glioma cells, which leads to cell apoptosis. NO production in glioma cells can alter many important pathophysiological processes, because chloroquine is effective in the treatment of diseases associated with increased secretion of pro-inflammatory cytokines such as malaria and rheumatoid arthritis; programmed cell death of inflammatory cells may underlie its anti-malarial and anti-rheumatic effects [25]. Thus, it can be proposed that both the therapeutic effects and the deleterious side effects of chloroquine may be caused by inflammatory cell apoptosis.

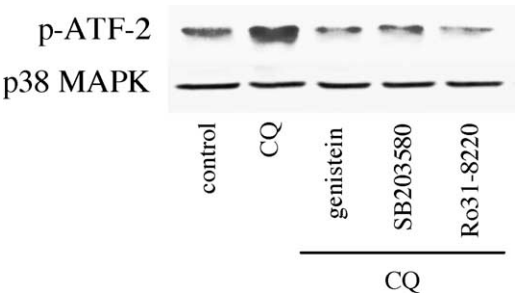

Fig. 5. Effects of SB 203580, genistein, and Ro 31-8220 on chloroquine-stimulated increases in p38 MAPK activity in C6 glioma cells. C6 glioma cells were pre-treated with genistein (20 μM), SB 203580 (10 μM), or Ro 31-8220 (3 μM) for 30 min before the addition of 100 μM chloroquine for 30 min. After incubation, cells were lysed, and p38 MAPK activity was determined with an immunocomplex kinase assay using ATF-2 as the substrate as described in Section 2.

Our results agree with the observation that iNOS is upregulated in chloroquine-treated mice [12]. Chloroquine increased NO production by increasing the NOS activity in murine, porcine, and human endothelial cells [7]. In contrast to the present observations, chloroquine inhibits interferon γ- and LPS-stimulated iNOS expression in macrophages [26,27] and LPS-induced IL-1β and TNF-α release in monoocytic THP-1 cells [28]. It has been shown that the inhibitory effect is due to a reduction in pro-inflammatory cytokine synthesis. Indeed, chloroquine inhibits the synthesis of tumor necrosis factor-α and interferon-γ in macrophages [27,29]. The inhibitory effect on macrophages by chloroquine was not due to a cytotoxic effect, because treatment with chloroquine (100 μM) for 24 h did not affect total cell counts, cell viability, or LDH release, which excludes the possibility of cell cytotoxicity [29,30].

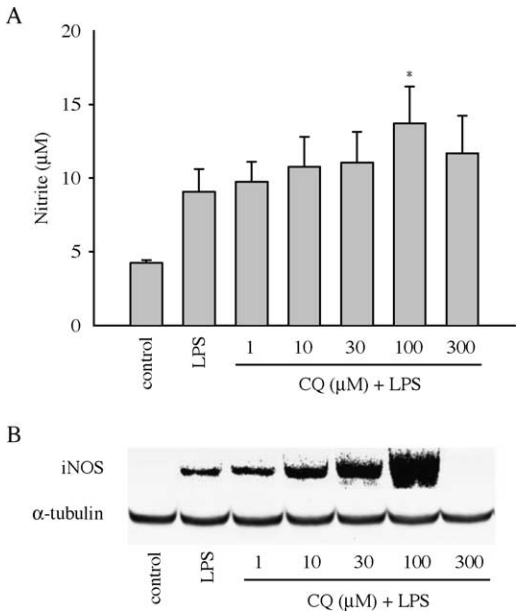

Fig. 6. Effects of chloroquine on nitrite accumulation and iNOS expression in LPS-treated C6 glioma cells. Cells were treated with LPS (0.5 μg ml^{-1}) in the presence or absence of various concentrations of chloroquine for 48 h, then the medium was removed and analyzed for nitrite accumulation (A). Data represent the mean ± S.E.M. of three independent experiments in triplicate. In (B), cells were incubated with LPS (0.5 μg ml^{-1}) in the presence or absence of various concentrations of chloroquine for 24 h, and then immunodetected with an iNOS-specific antibody as described in Section 2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin.

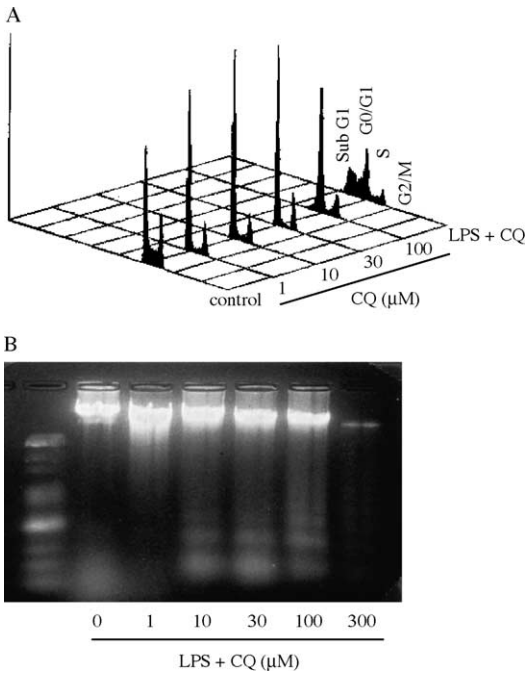

Fig. 7. Induction of programmed cell death by chloroquine in the presence or absence of LPS in C6 glioma cells. In (A), C6 glioma cells were incubated with different concentrations of chloroquine or with 100 μM of chloroquine in the presence LPS (0.5 μg ml^{-1}). DNA contents in different phases of cell cycle were analyzed using a fluorescence-activated cell sorter (FACS). In (B), C6 glioma cells were incubated with different concentrations of chloroquine in the presence of LPS (0.5 μg ml^{-1}) for 48 h. DNA fragmentation was examined as described in Section 2; the DNA was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Inhibition of chloroquine-stimulated iNOS expression and nitrite release by genistein suggests that the responses are also mediated by signaling through tyrosine phosphorylation. Although the identity of protein tyrosine kinase involved in the chloroquine-induced iNOS expression remained unclear, chloroquine has been shown to stimulate tyrosine phosphorylation of EGF receptor and src in EGF-stimulated cells [31], and increase phosphotyrosine content and protein tyrosine kinase activity in insulin-treated endosomes [32]. Consistent with our finding, LPS-stimulated iNOS expression is inhibited by the tyrosine kinase inhibitors in murine macrophages [33] and in retinal epithelial cells [34]. On the other hand, PKC has been shown to play an important role in regulation of NO production in microglia [35]. In agreement, we demonstrated that chloroquine stimulates PKC-α, -δ translocation in C6 glioma cells, and that Ro 31-8220, which specifically inhibits PKC -α, -β, -γ, and -ε, blocks the chloroquine-stimulated NO production in C6 glioma cells. The murine iNOS promoter contains 24 transcriptional factor binding sites [36]. Some of these transcription factors are regulated by p38 MAPK [37]. It is possible that different regulatory signals may converge into common pathways (i.e., p38 MAPK) and cause greater iNOS induction. In this study, we present evidence that chloroquine activates tyrosine kinase and protein kinase C, which in turn activates p38 MAPK and results in iNOS induction. Our results agree with the finding that p38 MAPK is involved in LPS-induced iNOS expression in C6 glioma cells [38]. The murine iNOS promoter also contains NF-κB binding sites. In human astrogial cells, NF-κB mediated chloroquine-induced CCL-2 and CXCL-8 chemokine expression [11]. However, chloroquine-induced iNOS expression was unaffected by PDTC, the inhibitor of NF-κB activation, suggesting that NF-κB is not involved in the signal transduction pathways by which iNOS is induced. These results differ from the iNOS induction stimulated by cytokines and LPS in C6 glioma cells [39]. Similarly, chloroquine-induced iNOS expression cannot be inhibited by pre-treatment with the MEK inhibitor, PD 98059, suggesting that p42/44 MAPK is not involved in iNOS induction in C6 glioma cells. However, given PDTC and PD98059 are not so selective to NF-κB and MEK-1, the involvement of these proteins may not necessarily be excluded.

We found that treatment of LPS-stimulated cells with chloroquine (100 μM) results in a significant loss of adherent C6 glioma cells, which appeared after a 48 h incubation. Regulation of programmed cell death is associated with many pathological conditions. For example, abnormal low apoptosis of inflammatory cells was noted in systemic lupus and rheumatoid arthritis. Apoptotic cell death as a result of iNOS activation comprising upregulation of the tumor suppressor, p53, caspase activation, chromatin condensation, and DNA fragmentation has been demonstrated in many cell types [40]. Results from this study suggest that chloroquine may selectively increases the apoptosis of LPS-stimulated cells through excessive NO production. Thus, chloroquine being a potent therapeutic agent in anti-malaria and anti-autoimmune diseases may be partly due to increased inflammatory cell apoptosis. In line with our findings, chloroquine killing of *Plasmodium falciparum* is associated with cell apoptosis [30]. Further, chloroquine at a concentration similar to that used in our report induced cell apoptosis in human endothelial cells [25] and in cultured hepatocytes [41]. In conclusion, chloroquine may activate tyrosine kinase and PKC pathways to induce p38 MAPK activation, which in turn induces iNOS expression and NO production in C6 glioma cells.

Acknowledgements

The work was supported by Grants NSC89-2320-B-038-009 and NSC90-2320-B-038-030 from the National Science Council, Taipei, Taiwan, ROC. The authors wish to thank Shu-Ting Tsai and Shiau-Ren Leu for their skilled technical assistance.

References

[1] Borba EF, Turrini-Filho JR, Kuruma KA, Bertola C, Pedalini ME, Lorenzi MC, et al. Chloroquine gestational use in systemic lupus erythematosus: assessing the risk of child ototoxicity by pure tone audiometry. Lupus 2004;13:223–7.

[2] Romanelli F, Smith KM, Hoven AD. Chloroquine and hydroxychloroquine as inhibitors of human immunodeficiency virus (HIV-1) activity. Curr Pharm Des 2004;10:2643–8.
[3] Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect Dis 2003;3:722–7.
[4] Weinblatt ME. Rheumatoid arthritis: more aggressive approach improves outlook. Cleve Clin J Med 2004;71:409–13.
[5] Wellems TE. How chloroquine works. Nature 1992;355:108–9.
[6] Fox R. Anti-malarial drugs: possible mechanisms of action in autoimmune disease and prospects for drug development. Lupus 1996;5:S4–10.
[7] Ghigo D, Aldier E, Todde R, Costamagna C, Garbarino G, Pescarmona G, et al. Chloroquine stimulates nitric oxide synthesis in murine, porcine, and human endothelial cells. J Clin Invest 1998;102:595–605.
[8] Good MI, Shader RI. Lethality and behavioral side effects of chloroquine. J Clin Psychopharmacol 1982;2:40–7.
[9] Klinghardt GW, Fredman P, Svennerholm L. Chloroquine intoxication induces ganglioside storage in nervous tissue: a chemical and histopathological study of brain, spinal cord, dorsal root ganglia, and retinal in the miniature pig. J Neurochem 1981;37:897–908.
[10] Ivy GO, Schottler F, Wenzel J, Baudry M, Lynch G. Inhibitors of lysosomal enzymes: accumulation of lipofuscin-like dense bodies in the brain. Science 1984;226:985–7.
[11] Park J, Choi K, Jeong E, Kwon D, Benveniste EN, Choi C. Reactive oxygen species mediate chloroquine-induced expression of chemokines by human astroglial cells. Glia 2004;47:9–20.
[12] Prada J, Muller S, Bienzle U, Kremsner PG. Upregulation of reactive oxygen and nitrogen intermediates in *Plasmodium berghei*-infected mice after rescue therapy with chloroquine. J Antimicrob Chemother 1996;38:95–102.
[13] Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 1994;298:249–58.
[14] Gross SS, Wolin MS. Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 1995;57:737–69.
[15] Li YM, Dickson DW. Enhanced binding of advanced glycation endproducts (AGE) by the ApoE4 isoform links the mechanism of plaque deposition in Alzheimer's disease. Neurosci Lett 1997;226:155–8.
[16] Horie K, Miyata T, Yasuda T, Takeda A, Yasuda Y, Maeda K, et al. Immunohistochemical localization of advanced glycation end products, pentosidine, and carboxymethyllysine in lipofuscin pigments of Alzheimer's disease and aged neurons. Biochem Biophys Res Commun 1997;236:327–32.
[17] Zimmerman GA, Meistrell 3rd M, Bloom O, Cockroft KM, Bianchi M, Risucci D, et al. Neurotoxicity of advanced glycation endproducts during focal stroke and neuroprotective effects of aminoguanidine. Proc Natl Acad Sci USA 1995;92:3744–8.
[18] Lee P, Lee J, Kim S, Lee MS, Yagita H, Kim SY, et al. NO as an autocrine mediator in the apoptosis of activated microglial cells: correlation between activation and apoptosis of microglial cells. Brain Res 2001;892:380–5.
[19] Shinoda J, Whittle IR. Nitric oxide and glioma: a target for novel therapy? Br J Neurosurg 2001;15:213–20.
[20] Munch G, Thome J, Foley P, Schinzel R, Riederer P. Advanced glycation endproducts in ageing and Alzheimer's disease. Brain Res Rev 1997;23:134–43.
[21] Gross SS, Jaffe EA, Levi R, Kilbourn RG. Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogues with a rank order of potency characteristic of activated macrophages. Biochem Biophys Res Commun 1991;178:823–9.
[22] Barna M, Komatsu T, Reiss CS. Activation of type III nitric oxide synthase in astrocytes following a neurotropic viral infection. Virology 1996;223:331–43.
[23] Lin CH, Lin YF, Chang MC, Wu CH, Ho YS, Lee HM. Advanced glycosylation end products induce nitric oxide synthase expression in C6 glioma cells: Involvement of a p38 MAP kinase-dependent mechanism. Life Sci 2001;69:2503–15.
[24] Chen CC, Chang J, Lin WW. Differential expression of protein kinase C isoforms in glial and neuronal cells. Translocation and down-regulation of PKC isoforms in C6 glioma and NG 108-15 hybrid cells: effects of extracellular Ca^{2+} depletion. Neurochem Int 1995;26:455–64.
[25] Potvin F, Petitclerc E, Marceau F, Poubelle PE. Mechansims of action of antimalarials in inflammation: induction of apoptosis in human endothelial cells. J Immunol 1997;158:1872–9.
[26] Hrabak A, Sefrioui H, Vercruysse V, Temesi A, Bahor T, Vray B. Action of chloroquine on nitric oxide production and parasite killing by macrophages. Eur J Pharmacol 1998;354:83–90.
[27] Park YC, Pae HO, Yoo JC, Choi BM, Jue DM, Chung HT. Chloroquine inhibits inducible nitric oxide synthase expression in murine peritoneal macrophages. Pharm Toxicol 1999;85:188–91.
[28] Seitz M, Valbracht J, Quach J, Lotz M. Gold sodium thiomalate and chloroquine inhibit cytokine production in monocytic THP-1 cells through distinct transcriptional and posttranslational mechanisms. J Clin Immunol 2003;23:477–84.
[29] Karres I, Kremer JP, Dietl I, Steckholzer U, Jochum M, Ertel W. Chloroquine inhibits proinflammatory cytokine release into whole blood. Am J Physiol 1998;274:R1058–64.
[30] Picot S, Burnod J, Bracchi V, Chumpitazi BF, Ambroise-Thomas P. Apoptosis related to chloroquine sensitivity of the human malaria parasite *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 1997;91:590–1.
[31] Oksvold MP, Skarpen E, Wierod L, Paulsen RE, Huitfeldt HS. Relocalization of activated EGF receptor and its signal transducers to multivesicular compartments downstream of early endosomes in response to EGF. Eur J Cell Biol 2001;80:285–94.
[32] Bevan AP, Krook A, Tikerpae J, Seabright PJ, Siddle K, Smith GD. Chloroquine extends the lifetime of the activated insulin receptor complex in endosomes. J Biol Chem 1997;272:26833–40.
[33] Dong Z, Qi X, Xie K, Fidler IJ. Protein tyrosine kinase inhibitors decrease induction of nitric oxide synthase activity in lipopolysaccharide-responsive and lipopolysaccharide-nonresponsive murine macrophages. J Immunol 1993;151:2717–24.
[34] Faure V, Courtois Y, Goureau O. Tyrosine kinase inhibitors and antioxidants modulate NF-κB and NOS-II induction in retinal epithelial cells. Am J Physiol 1998;275:C208–15.
[35] Nakamura Y, Si Q, Kataoka K. Differential regulation of microglial NO production by protein kinase C inhibitors. Neurochem Int 2001;38:1–7.
[36] Xie QW, Wishnan R, Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. J Exp Med 1993;177:1779–84.
[37] Karin M. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. Curr Opin Cell Biol 1994;6:415–24.
[38] Xu X, Malave A. P38 MAPK, but not p42/p44 MAPK, mediated inducible nitric oxide synthase expression in C6 glioma cells. Life Sci 2000;67:3221–30.
[39] Nishiya T, Uehara T, Kaneko M, Nomura Y. Involvement of nuclear factor-kappaB (NF-kappaB) signaling in the expression of inducible nitric oxide synthase (iNOS) gene in rat C6 glioma cells. Biochem Biophys Res Commun 2000;275:268–73.
[40] Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. Eur J Pharmacol 1998;351:261–72.
[41] Maeda S, Lin KH, Inagaki H, Saito T. Induction of apoptosis in primary culture of rat hepatocytes by protease inhibitors. Biochem Mol Biol Int 1996;39:447–53.