

Suppression of Japanese encephalitis virus infection by non-steroidal anti-inflammatory drugs

Chun-Jung Chen,¹ Shue-Ling Raung,¹ Ming-Der Kuo² and Yu-Ming Wang²

¹ Department of Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan, Republic of China

² Institute of Preventive Medicine, National Defence Medical Center, Taipei, Taiwan, Republic of China

Japanese encephalitis virus (JEV) infection generates a rapid inflammatory response including peripheral neutrophil leucocytosis and infiltration of neutrophils into extraneural tissue. The level of inflammation correlates well with the clinical outcome in Japanese encephalitis patients. Non-steroidal anti-inflammatory drugs (NSAIDs), used medicinally for their analgesic and anti-inflammatory properties, are being considered for prevention of cardiovascular disease and cancer, as well as for treatment of human immunodeficiency virus infection. Apart from their ability to inhibit prostaglandin synthesis, the mechanisms underlying the beneficial therapeutic effects are largely unknown. We used aspirin, indomethacin and sodium salicylate to study the role of NSAIDs in JEV propagation *in vitro*. We found that NSAIDs suppressed JEV propagation in neuronal and non-neuronal cells. Blockade of cyclooxygenase activity by NSAIDs caused decreased production of free radicals and prostaglandins. However, these pharmacological alterations did not seem to correlate well with the antiviral effects. When cells were treated with the mitogen-activated protein kinase (MAPK) inhibitors PD 98059 and SB 203580, salicylate lost its antiviral effect. The activation of MAPK by anisomycin mimicked the action of salicylate in suppressing JEV-induced cytotoxicity. The decreased phosphorylation of extracellular signal-regulated kinase (ERK) was induced by JEV infection and the decrease in ERK was reversed by salicylate. Our data suggest that the signalling pathways of MAPK play a role in the antiviral action of salicylate.

Introduction

Flaviviruses are important human pathogens causing a variety of diseases ranging from mild febrile illnesses to severe encephalitis and haemorrhagic fever. Among them, Japanese encephalitis virus (JEV) is an acute zoonotic infection that commonly affects children and is a major cause of acute encephalopathy (Chambers *et al.*, 1990). JEV targets the central nervous system (CNS), clinically manifesting with fever, headache, vomiting, signs of meningeal irritation and altered consciousness leading to high mortality and neurological sequelae in some of those who survive (Kumar *et al.*, 1990). After entry into the host, JEV generates a rapid inflammatory response, including peripheral neutrophil leucocytosis and infiltration of neutrophils into extraneural tissue (Chaturvedi *et al.*, 1979; Mathur *et al.*, 1988). The inflammation results in an

increased level of cytokines such as macrophage-derived chemotactic factor (MDF), tumour necrosis factor alpha (TNF- α) and interleukin 8 (IL-8) in the serum and cerebrospinal fluid (CSF) (Khanna *et al.*, 1991; Ravi *et al.*, 1997; Singh *et al.*, 2000). The increased levels of inflammatory mediators appear to play a protective role or to initiate an irreversible immune response leading to cell death. Accumulating evidence shows that the mortality rate increases with increasing concentrations of cytokines in the serum and CSF in Japanese encephalitis patients (Ravi *et al.*, 1997).

Non-steroidal anti-inflammatory drugs (NSAIDs) have long been used to treat fever and inflammatory diseases. Traditional NSAIDs like aspirin, ibuprofen and indomethacin can inhibit the activity of both cyclooxygenase (COX) isoforms, COX-1 and COX-2, thereby blocking the production of prostaglandins (Vane, 1971). Aspirin is a pro-drug form of salicylate, which is rapidly hydrolysed to salicylate *in vivo* (Gilman *et al.*, 1990). Salicylate, however, inhibits the synthesis of prostaglandins *in vivo*, but has little effect on COX1 and

Author for correspondence: Chun-Jung Chen.

Fax +886 4 23592705. e-mail cjchen@vghtc.vghtc.gov.tw

COX2 activities *in vitro* (Gilman *et al.*, 1990). Salicylate must therefore modulate the synthesis of prostaglandins through an alternate mechanism not involving direct effects on COXs. In addition to the anti-inflammatory action of salicylate, increasing evidence indicates its ability to protect against cancer, cardiovascular disease and viral infection (Huang & Dietsch, 1988; Primache *et al.*, 1998; Speir *et al.*, 1998; Chen *et al.*, 2000; Patrignani, 2000). This suggests that some of the biochemical effects of salicylate are independent of effects on COX activity.

The antiviral effects of NSAIDs have been shown to exert an influence on the influenza virus, cytomegalovirus and varicella-zoster virus (Huang & Dietsch, 1988; Primache *et al.*, 1998; Speir *et al.*, 1998). Inhibition of COX activity, scavenging of free radicals and down-regulation of transcription factors may be attributed to the antiviral action of NSAIDs. Despite extensive studies of NSAIDs, little is known about their effects on viral infection in the CNS. The propagation of vesicular stomatitis virus (VSV) in mouse CNS is suppressed by NSAIDs leading to a decrease in encephalitis (Chen *et al.*, 2000). Moreover, arachidonic acid and its metabolites are involved in the death of neuroblastoma cells induced by Dengue virus, one of the flaviviruses (Jan *et al.*, 2000). In the present study, we explored the effects of NSAIDs on JEV infection. We found that salicylate suppressed JEV replication and its cytotoxicity in neuroblastoma cells. The mechanism of its antiviral action was shown to be by modulation of the mitogen-activated protein kinase (MAPK) signalling pathway, not by inhibition of COX activity.

Methods

■ **Virus and cells.** A local Taiwanese strain of JEV NT113, isolated from mosquito, was generously provided by the National Institute of Preventive Medicine, Taiwan (Chen *et al.*, 1997). Virus propagation was carried out in C6/36 cells in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal bovine serum (FBS). N18 cells, a mouse neuroblastoma cell line, and baby hamster kidney (BHK21) cells were grown in DMEM containing 10% FBS.

■ **Virus infection and titre determination.** For infection of cells with JEV, monolayers of each cell type were first adsorbed with JEV at an m.o.i. of 5 for 1 h at 37 °C. After adsorption, unbound virus was removed by gentle washing with PBS, pH 7.4. Fresh medium was added to each plate for further incubation at 37 °C. To determine virus titres, culture media were harvested and used in plaque-forming assays. Briefly, various dilutions of the culture media were added to 80% confluent BHK21 cells and incubated at 37 °C for 1 h. After adsorption, the cells were washed and overlaid with 1% agarose (SeaPlaque; FMC BioProducts) in RPMI 1640 plus 2% FBS. After incubation for 4 days, the cells were fixed with 10% formaldehyde, stained with 0.5% crystal violet and examined for the presence of plaques.

■ **Cytotoxicity assessment.** Cytotoxicity, as indicated by cell membrane integrity, was assessed by measuring the activity of lactate dehydrogenase (LDH) in the cultured media by the colorimetric detection of formazan, using an LDH diagnostic kit (Promega). After each experiment, the supernatants were transferred to a microtitre plate and

incubated with reaction mixture at room temperature for 30 min for colour development. The OD₄₉₂ was measured using a spectrophotometer (PowerWaveX 340, BIO-TEK Instruments).

■ **MTT assay.** An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay was used to measure mitochondrial function as an index of living cells. In brief, MTT (Sigma) was dissolved in 0.1 M TBS to make a 5 mg/ml solution. Fifty µl of MTT solution was added to each well containing tested cells in a 12-well plate and incubated at 37 °C for 4 h. After incubation, the MTT solution was removed and 500 µl isopropanol containing 0.04 M HCl was added to dissolve the dark-blue crystals precipitated in the wells. A 100 µl sample of the resulting solution was removed from each well and the absorbance at 540 nm was determined using a microplate reader (PowerWaveX 340, Bio-Tek Instruments).

■ **Assessment of intracellular redox potential.** Levels of intracellular free radicals were analysed by the fluorescent signal produced after the oxidation of non-fluorescent 2',7'-dichlorofluorescein (Molecular Probe) by reactive oxygen species (ROS), as described previously (Zhu *et al.*, 1994). 2',7'-Dichlorofluorescein was added to cultures to a concentration of 5 µM in a defined salt buffer (116 mM NaCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 5.4 mM KCl, 1 mM NaH₂PO₄, 14.7 mM NaHCO₃, 10 mM HEPES and 5.5 mM glucose) at 37 °C for 5 min, washed and then incubated with buffer for 60 min. The fluorescent signal resulting from oxidation of 2',7'-dichlorofluorescein was observed using a fluorescent microscope (Leica; excitation 488 nm and emission 510 nm) or measured using a fluorometer (Fluoroskan Ascent, Labsystems).

■ **TdT-mediated dUTP nick end-labelling (TUNEL).** Apoptosis-induced DNA strand breaks were end-labelled with dUTP by use of terminal deoxynucleotidyltransferase (TdT) with a commercial kit (In Situ Cell Death Detection Kit, Boehringer Mannheim), according to the manufacturer's instructions. Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized in 0.1% Triton X-100–0.1% sodium citrate for 2 min on ice. The reaction was performed by labelling with fluorescein isothiocyanate–dUTP at 37 °C for 60 min, then incubated with an alkaline phosphatase-conjugated secondary antibody. The colour was developed by incubation with NBT/BCIP substrate and observed under a light microscope.

■ **Western blot.** Protein extracts (100 µg) were resolved by SDS–polyacrylamide gel electrophoresis and transferred on to a blotting membrane. The membrane was first incubated with 5% (w/v) skimmed milk in PBS for 30 min to reduce non-specific binding, then incubated with primary antibody overnight at 4 °C, followed by washing with 0.05% Tween 20 in PBS. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. The signals were developed by chemiluminescent detection. The intensity of signals was determined by a computer image system (Alpha Innotech Corporation, IS1000).

■ **Determination of prostaglandin E₂ (PGE₂) concentration.** PGE₂ levels were detected by ELISA, following the instructions provided by the manufacturer (Cayman Chemical).

■ **Northern blot.** Isolation of total cellular RNA and Northern blot analysis were performed as previously described (Chang *et al.*, 1994). The probe used was the antigenomic strand of JEV RNA (nt 10392–10976), synthesized *in vitro* with Sp6 RNA polymerase in the presence of Bio-11-UTP (Sigma). The signals were developed by chemiluminescent detection. The intensity of signals was determined by a computer image system (Alpha Innotech Corporation, IS1000). The 18S and 28S rRNA were detected by staining with ethidium bromide before probing.

■ **Statistical analysis.** Data are expressed as mean \pm SEM. For comparisons, the statistical significance between means was determined using one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A level of $P < 0.05$ was considered statistically significant.

Results

Cells treated with NSAIDs had lower viral production and cytotoxicity

The cytopathic effect of JEV infection in N18 cells became apparent 32–48 h post-infection (p.i.), concurrent with the period in which the infected cells actively produced large quantities of virus. The cytopathic effect resulting in cell death was demonstrated by the efflux of LDH and the presence of chromosomal DNA fragmentation. To investigate whether NSAIDs had an effect on JEV propagation, we treated cells with either a non-selective cyclooxygenase (COX) inhibitor

(aspirin), a selective COX-2 antagonist (indomethacin) or an aspirin derivative (sodium salicylate), after 1 h of virus adsorption. Cytotoxicity was assessed 40 h p.i. All three pharmacological agents attenuated JEV-induced LDH efflux, in a concentration-dependent manner (Fig. 1A). To determine whether chromosomal DNA breaks generated by JEV infection could be affected by NSAIDs, cells were labelled and analysed by TUNEL assay and microscopy for the presence of DNA fragmentation in the nuclei. After 32 h, JEV induced apoptotic cell death in 37–45% of N18 cells as indicated by TUNEL assay (Fig. 1B). JEV-induced apoptosis was decreased in the cells treated with sodium salicylate (21–28%, $P < 0.01$) and the other NSAIDs (data not shown). Furthermore, analysis of cellular viral protein expression (Fig. 1C) and viral titres (Fig. 1D) revealed that NSAID-treated cells produced less viral protein and virus compared with the control JEV-infected cells.

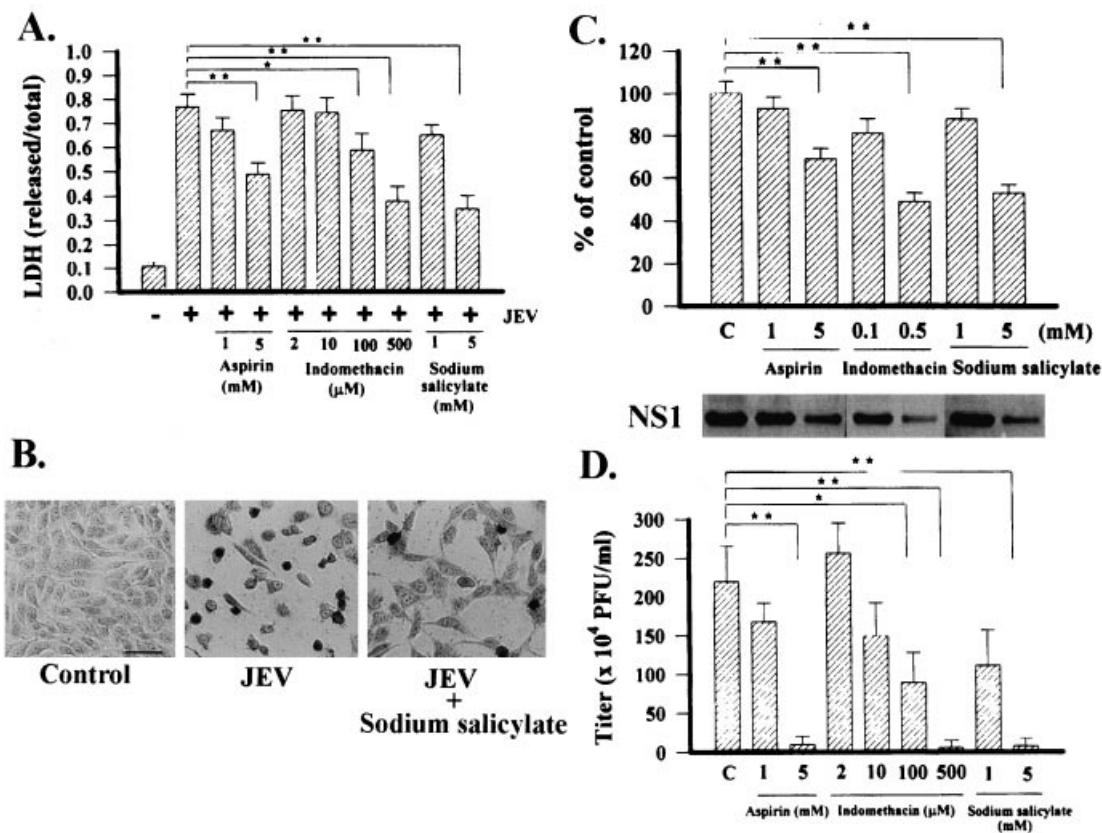


Fig. 1. NSAIDs suppress JEV propagation. (A) After 1 h of virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 40 h in the presence of aspirin (1 and 5 mM), indomethacin (2, 10, 100 and 500 μ M), or sodium salicylate (1 and 5 mM). Cell damage was assessed by measurement of LDH efflux ($n = 3$). $*P < 0.05$; $**P < 0.01$. (B) After 1 h of mock (control) or virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 32 h in medium alone (JEV), or in the presence of sodium salicylate (5 mM). Apoptotic cell death was examined by TUNEL assay and observed under a light microscope. Scale bar, 50 μ m. (C) After 1 h of virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 24 h in the presence of aspirin (1 and 5 mM), indomethacin (0.1 and 0.5 mM) or sodium salicylate (1 and 5 mM), or with medium only (control). Western blot analysis of the JEV NS1 protein was performed using cell lysates. The intensity of the NS1 signal in control cells was defined as 100% ($n = 3$). $**P < 0.01$. (D) After 1 h of virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 24 h in the presence of aspirin (1 and 5 mM), indomethacin (2, 10, 100 and 500 μ M) or sodium salicylate (1 and 5 mM), or with medium only (control). The JEV titres in the culture supernatants were measured by plaque assay ($n = 4$). $*P < 0.05$; $**P < 0.01$.

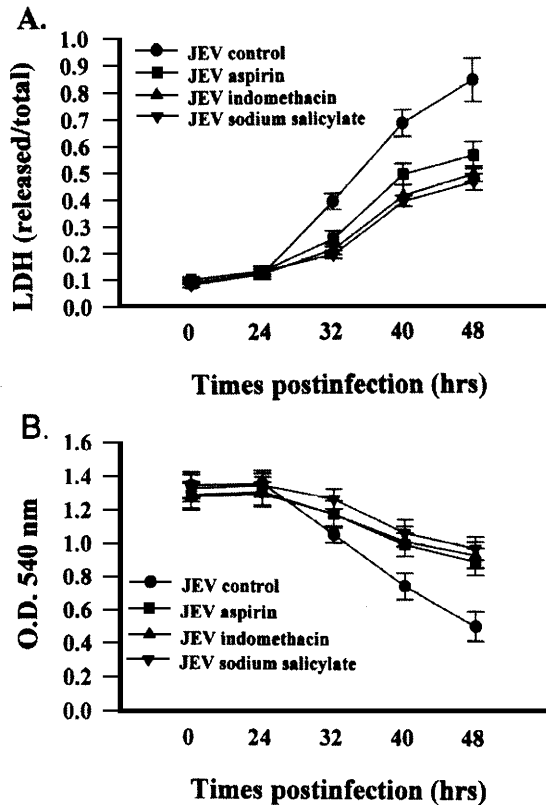


Fig. 2. Kinetics of the antiviral effects of NSAIDs. After 1 h of virus adsorption (m.o.i. = 5), N18 cells were cultivated for up to 48 h in the absence (control) or presence of either aspirin (5 mM), indomethacin (500 μ M) or sodium salicylate (5 mM). Cell damage was assessed by (A) measurement of LDH efflux ($n = 3$) and (B) MTT assay ($n = 3$).

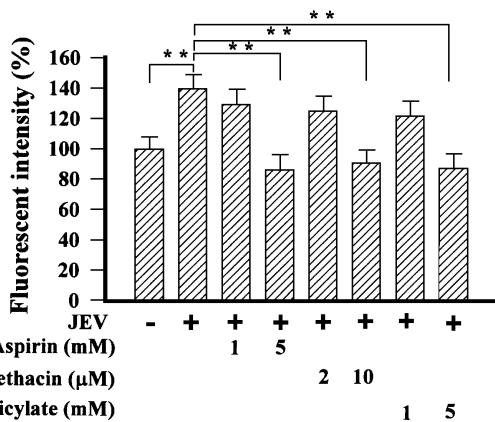


Fig. 3. NSAIDs reduce JEV-induced intracellular redox potential change. After 1 h of mock or virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 24 h in the presence of aspirin (1 and 5 mM), indomethacin (2 and 10 μ M) or sodium salicylate (1 and 5 mM). Intracellular redox change was indicated by the fluorescent signal produced by 2',7'-dichlorofluorescein (5 μ M) oxidation. The fluorescent intensity of uninfected control cells was defined as 100%. ** $P < 0.01$; $n = 3$.

In addition, the protective effects of NSAIDs against JEV-mediated cytotoxicity were assessed kinetically. NSAIDs suppressed JEV-induced LDH efflux ($P < 0.01$, Fig. 2A) and

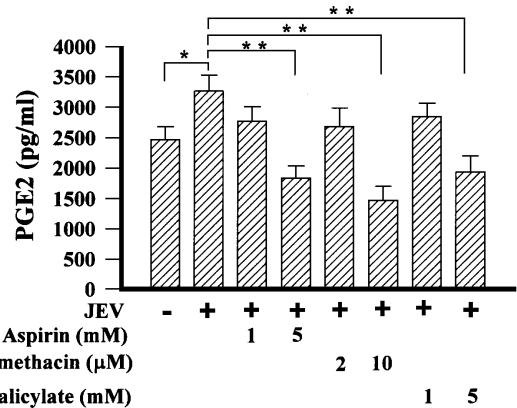


Fig. 4. NSAIDs reduce JEV-induced PGE₂ release. After 1 h of mock or virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 24 h in the presence of aspirin (1 and 5 mM), indomethacin (2 and 10 μ M) or sodium salicylate (1 and 5 mM). The release of PGE₂ in the culture supernatants was measured by ELISA. * $P < 0.05$; ** $P < 0.01$; $n = 3$.

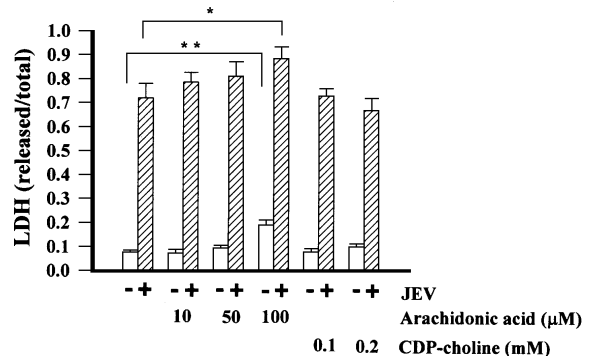


Fig. 5. Effects of arachidonic acid and PLA₂ inhibitor on JEV-induced LDH efflux. After 1 h of mock or virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 40 h in the presence of arachidonic acid (10, 50 and 100 μ M) or CDP-choline (0.1 and 0.2 mM), a PLA₂ inhibitor. Cell damage was assessed by measurement of LDH efflux. * $P < 0.05$; ** $P < 0.01$; $n = 3$.

JEV-induced reduction of MTT reaction ($P < 0.01$, Fig. 2B), in a time-dependent manner. Taken together, NSAID treatment suppresses JEV replication and propagation in neuroblastoma cells and leads to the attenuation of virus-induced cytotoxicity.

Effects of NSAID treatment on virus-induced redox potential change and prostaglandin release

We have previously shown that JEV infection of neuroblastoma cells generates intracellular reactive oxygen species (ROS), which can be assessed by quantification of the fluorescent signals produced by the oxidation of 2',7'-dichlorofluorescein (Raung *et al.*, 2001). Both aspirin and indomethacin diminished JEV-induced ROS generation in a concentration-dependent manner (Fig. 3). This effect was not entirely due to the inhibition of COX enzymes since sodium

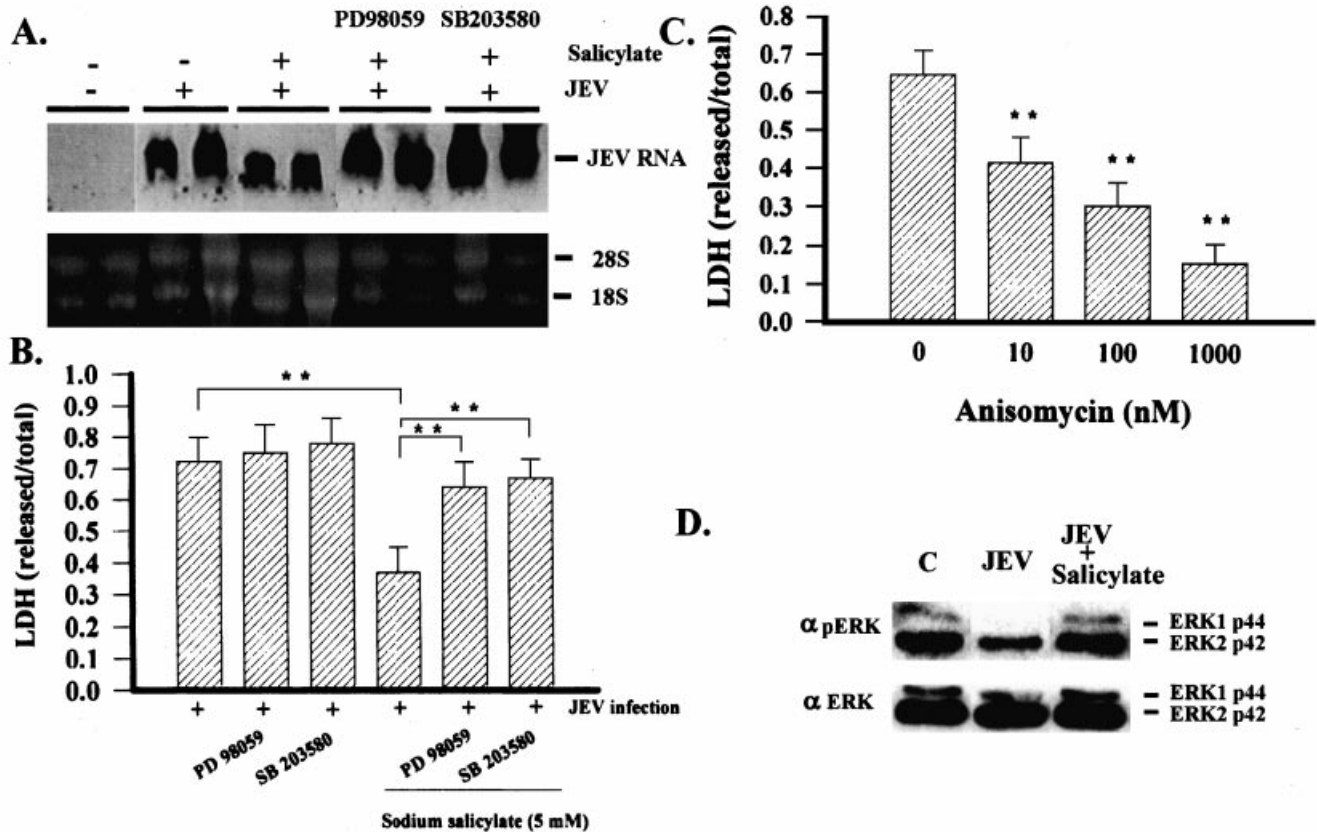


Fig. 6. Antiviral effects of salicylate are antagonized by inhibitors of MAPK. (A) After 1 h of mock or virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 24 h in the presence of sodium salicylate (5 mM) or sodium salicylate plus PD 98059 (25 μ M) or SB 203580 (25 μ M). Total cellular RNA was isolated and the presence of genomic viral RNA was detected by Northern blot (upper panel). Ethidium bromide staining before gel transfer detected ribosomal RNAs (lower panel). (B) After 1 h of virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 40 h in the presence of sodium salicylate (5 mM) or medium alone, with or without either PD 98059 (25 μ M) or SB 203580 (25 μ M). Cell damage was assessed by measurement of LDH efflux ($n = 3$). ** $P < 0.01$. (C) After 1 h of virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 40 h in the absence or presence of anisomycin (10, 100 and 1000 nM). Cell damage was assessed by measurement of LDH efflux ($n = 3$). ** $P < 0.01$. (D) After 1 h of mock (control) or virus adsorption (m.o.i. = 5), N18 cells were cultivated for a further 24 h in the presence of sodium salicylate (5 mM). Western blot analysis for detection of ERK or phospho-ERK was performed using cell lysates.

salicylate also diminished ROS generation (Fig. 3). This compound has only minimal COX inhibitory activity (Weissmann, 1991) but, like aspirin and indomethacin, is a potent ROS scavenger (Sagone & Husney, 1987). To further characterize the involvement of COX enzymes in NSAID-mediated antiviral action, we assessed the COX activity as measured by PGE₂ production. At 24 h after infection of N18 cells with JEV, there was a considerably higher level of PGE₂ in the medium compared with the control medium containing uninfected cells (Fig. 4). We found that aspirin, indomethacin and even sodium salicylate decreased PGE₂ release in a concentration-dependent manner (Fig. 4). These results indicate that aspirin, indomethacin and sodium salicylate do attenuate JEV-induced ROS generation and PGE₂ production. However, these actions did not correlate well with their antiviral effect because the antioxidant treatments failed to suppress JEV-induced cytotoxicity (Raung *et al.*, 2001) and the concentra-

tions that exerted an antiviral effect were higher than those needed to diminish ROS generation and PGE₂ release.

Effect of arachidonic acid (AA) on viral cytotoxicity

COX inhibitors are reported to cause AA accumulation by blocking the catabolism of AA (Axelrod *et al.*, 1988; Kalgutkar *et al.*, 1998). AA, a lipid secondary messenger, is generated by hydrolysis of membrane phospholipids via phospholipase A₂ (PLA₂). We found that cytidine-5'-diphosphocholine (CDP-choline), a phospholipase A₂ inhibitor, did not affect JEV-induced cytotoxicity (Fig. 5). Moreover, the addition of AA was ineffective in preventing JEV-induced cytotoxicity. Instead, AA increased JEV-induced LDH efflux in a concentration-dependent manner (Fig. 5). Exogenous AA itself also caused cell damage at a concentration of 100 μ M (Fig. 5). These results strongly suggest that AA does not play a role in the antiviral action of NSAIDs.

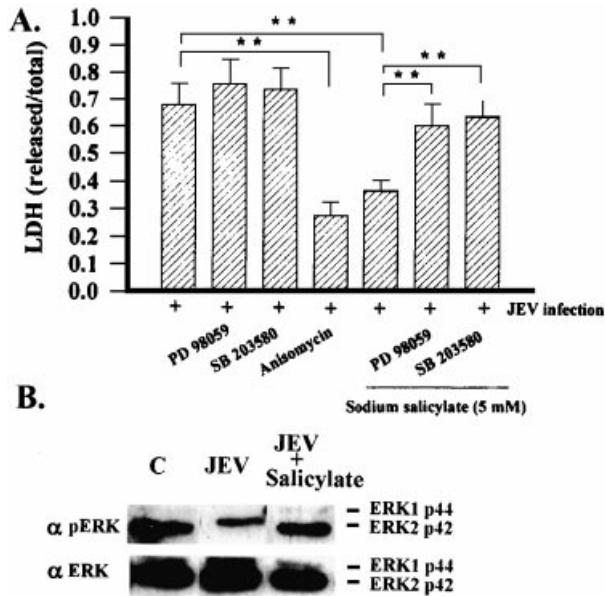


Fig. 7. Salicylate suppresses JEV propagation in non-neuronal cells. (A) After 1 h of virus adsorption (m.o.i. = 5), BHK21 cells were cultivated for a further 40 h in the presence of sodium salicylate (5 mM) or medium alone, with or without either PD 98059 (25 μ M) or SB 203580 (25 μ M), or with anisomycin (100 nM) only. Cell damage was assessed by measurement of LDH efflux ($n = 3$). $**P < 0.01$. (B) After 1 h of mock (control) or virus adsorption (m.o.i. = 5), BHK21 cells were cultivated for a further 24 h in the presence of sodium salicylate (5 mM). Western blot analysis for detection of ERK or phospho-ERK was performed using cell lysates.

Inhibition of mitogen-activated protein kinase (MAPK) activation reverses the effect of sodium salicylate on viral growth

The MAPK cascades have an important role in cell growth, differentiation, survival and apoptosis. Recent evidence indicates that sodium salicylate is able to activate MAPK (Schwenger *et al.*, 1998). Therefore, we wanted to determine whether the antiviral action of sodium salicylate was through the modulation of MAPK activation. Sodium salicylate repressed the replication of JEV (71% of wild-type control, $P < 0.05$) as indicated by the decreased detection of genomic viral RNA (Fig. 6A). Furthermore, we found that both of the MAPK inhibitors, PD 98059 and SB 203580, reversed the negative effect of sodium salicylate on JEV RNA replication (119% and 115% of wild-type control, respectively; Fig. 6A). The preventive action of MAPK inhibitors was also apparent in sodium salicylate-mediated reduction of JEV-induced LDH efflux (Fig. 6B). Moreover, activation of MAPK by anisomycin attenuated JEV-induced LDH efflux in a concentration-dependent manner (Fig. 6C). These results suggest that activation of MAPK suppresses JEV-induced cytotoxicity. Indeed, infection of neuroblastoma cells with JEV resulted in decreased phosphorylation (41% of wild-type control, $P < 0.01$) of extracellular signal-regulated kinase (ERK). This down-regulation of ERK phosphorylation was partially reversed by

sodium salicylate (79% of wild-type control, $P < 0.01$) (Fig. 6D). Moreover, the suppressive effects of aspirin and indomethacin against JEV-induced cytotoxicity were also reversed by blocking MAPK activation with PD 98059 and SB 203580 (data not shown).

Sodium salicylate suppresses JEV-induced cytotoxicity in non-neuronal cells

Sodium salicylate suppressed JEV-induced cytotoxicity in murine neuroblastoma cells (Figs 1 and 6). The antiviral action of sodium salicylate was investigated in non-neuronal cells infected with JEV. Similar to the results in neuroblastoma cells, sodium salicylate was shown to diminish JEV-induced cytotoxicity in BHK21 cells (Fig. 7).

Discussion

NSAID-treated cells are more resistant to JEV-induced cytotoxicity. Both the non-selective and selective COX inhibitors, aspirin, indomethacin and sodium salicylate, reduced virus replication and yield *in vitro*. These antagonists suppressed JEV-induced intracellular redox potential change and PGE₂ production. The antiviral action of salicylate is independent of the inhibition of COX activity, since the doses used to suppress JEV amplification are higher than those required to inhibit prostaglandin synthesis. In addition, COX activity inhibition-induced AA accumulation failed to eliminate viral toxicity. Instead, MAPK inhibitors reversed the antiviral effect of salicylate. Taken together, these results show that salicylate suppresses JEV propagation *in vitro* through the modulation of the MAPK signalling pathway.

Phospholipids play roles in many biological processes, including the generation of proinflammatory lipid mediators, such as prostaglandins and leukotrienes, and the regulation of lipid metabolism (Glaser, 1995). The mobilization of AA from phospholipids requires the involvement of PLA₂. AA serves as a substrate for COX enzymes, resulting in the production of prostaglandins. Prostaglandins activate cellular receptors, resulting in the subsequent initiation of signal cascades involving G-protein and cyclic AMP (Cirino, 1998). These signalling cascades have an important role in cellular injury via their ability to mediate inflammatory responses. NSAIDs inhibit the activity of both COX isoforms, leading to decreased production of prostaglandins and the accumulation of AA. Therefore, the inflammatory action of prostaglandins and COX activity-associated free-radical generation are suppressed. In contrast, the biological action of AA is apparent.

NSAIDs such as aspirin, indomethacin and sodium salicylate suppressed JEV propagation in neuroblastoma cells, as indicated by the attenuation of cell death and the reduction of viral protein expression and virus yield (Figs 1 and 2). Numerous studies have used PGE₂ release as a marker for COX activity. JEV infection slightly increased the production of PGE₂ by neuroblastoma cells (Fig. 4). These NSAIDs sup-

pressed mock-infection (data not shown) and JEV-induced PGE₂ production, indicating the well-known pharmacological effects on COX enzymes. Previously, we reported that JEV infection caused generation of free radicals in neuroblastoma cells via the involvement of the flavon-containing enzymes (Raung *et al.*, 2001). The increased production of free radicals induced by JEV could be eliminated by NSAIDs (Fig. 3). This result suggests that JEV-induced free-radical generation might be through the COX-associated pathway. However, it should be noted that the reduction of free radicals may be derived from the direct scavenging effect. Recently, Jan *et al.* (2000) reported that AA, superoxide anion and nuclear factor kappa B (NF- κ B) were sequentially involved in Dengue virus-triggered apoptotic pathways. However, exogenous AA and an inhibitor of PLA₂ failed to mimic the action of NSAIDs in suppressing JEV-induced cell death (Fig. 5). Although reduction of superoxide anion generation by superoxide dismutase protected Dengue virus-induced apoptosis (Jan *et al.*, 2000), antioxidants showed no protective actions against JEV-mediated cytotoxicity (Raung *et al.*, 2001). Moreover, the doses of NSAIDs used to eliminate free-radical generation (Fig. 3) and PGE₂ production (Fig. 4) were lower than those required to suppress JEV propagation (Figs 1–4). Therefore, these results suggest that the antiviral effect of NSAIDs was not through the conventional COX pathway.

Signal transduction pathways convey signals generated at the cell surface into the cell nucleus in order to initiate a programme of gene expression that is characteristic for particular stimuli. Among them, MAPKs, including ERK, p38 protein kinase and c-Jun N-terminal kinase (JNK), play a critical role in the regulation of cell proliferation and differentiation in response to mitogens and other extracellular stimuli (Karin, 1998). The activation of MAPKs can be modulated by salicylate (Schwenger *et al.*, 1998). We found that the antiviral effect of salicylate against JEV could be blocked by inhibitors of mitogen-activated protein kinase (MEK) (PD 98059) or p38 MAPK (SB 203580) (Fig. 6). In addition, direct activation of p38 MAPK by anisomycin also markedly suppressed JEV-induced LDH efflux (Fig. 6). The involvement of MAPK modulation on the antiviral effect of salicylate was observed not only in neuroblastoma cells but also in non-neuronal BHK21 cells (Fig. 7). Recently, Liao *et al.* (2001) have demonstrated that the antinflavirus effect of salicylates was partially reversed by blocking p38 MAPK activation with SB 203580 in BHK21 cells. In this study, we have provided further evidence showing the involvement of p38 MAPK and ERK in JEV-mediated cytotoxicity as well as in the antiviral effect of salicylates. In general, the dynamic balance between branches of the MAPK family is believed to regulate neuronal decisions to live or die in response to stressors (Xia *et al.*, 1995). In particular, ERK activation may play a pivotal role. For example, many neuroprotective/neurotrophic factors activate receptor tyrosine kinases transmitting signals through the activation of ERK (Segal & Greenberg, 1996). ERK activation appears to

antagonize apoptotic pathways in some cell systems (Xia *et al.*, 1995). In contrast, several recent studies indicate that ERK activation may also play a pathologic role in neurons exposed to increased oxidative stress (Oh-hashii *et al.*, 1999; Stanciu *et al.*, 2000). Although the detailed mechanisms underlying the antiviral effect remain unclear, JEV infection-induced decreased phosphorylation of ERK could be reversed by salicylate (Fig. 6). In addition, the activation of p38 MAPK also suppressed JEV-induced toxicity (Fig. 6). Thus, the activation of MAPK plays a role in host-cell protection against JEV infection.

Oxidative stress has been suggested to be a mediator of apoptosis/necrosis induced by a variety of triggers, including virus infections (Schwarz, 1996; Peterhans, 1997; Speir *et al.*, 1998; Schweizer & Peterhans, 1999). Some antioxidants are known to inhibit cell death in virus systems (Cossarizza *et al.*, 1995; Verhaegen *et al.*, 1995; Lowy & Dimitrov, 1997), although antioxidants do not protect cells against JEV-induced toxicity (Raung *et al.*, 2001). Free radicals are potent triggers for NF- κ B activation. Accumulating evidence indicates the antiviral effects of NF- κ B inhibition in cytomegalovirus, Dengue virus, respiratory syncytial virus and human immunodeficiency virus (Schwarz, 1996; Bitko *et al.*, 1997; Peterhans, 1997; Speir *et al.*, 1998; Jan *et al.*, 2000). In these viruses, antioxidant therapy is effective against virus infection (Staal *et al.*, 1990; Schwarz, 1996; Bitko *et al.*, 1997; Peterhans, 1997; Speir *et al.*, 1998; Jan *et al.*, 2000). NF- κ B inhibition has been shown to be a potential mechanism of the neuroprotection effect exerted by aspirin and salicylate (Grilli *et al.*, 1996). However, both antioxidants (Raung *et al.*, 2001) and the inactivation of NF- κ B (Liao *et al.*, 2001) failed to attenuate JEV propagation and JEV-mediated cytotoxicity. Therefore, the inactivation of NF- κ B is not involved in the antiviral effect of salicylate against JEV.

The generation of ROS has been demonstrated to be critical in the rapid degradation of phagocytosed JE viral protein and nucleic acid (Srivastava *et al.*, 1999). In addition, nitric oxide appears to be effective in restricting JE amplification (Lin *et al.*, 1997). Usually, oxidative stress is well associated with the activation of MAPK. In this study, we have demonstrated that MAPK signalling pathways are also involved in suppression of JEV propagation.

This work was supported by grant TCVGH-907307C from Taichung Veterans General Hospital, Taiwan, Republic of China.

References

- Axelrod, J., Burch, R. M. & Jelsema, C. L. (1988).** Receptor-mediated activation of phospholipase A₂ via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends in Neuroscience* **11**, 117–123.
- Bitko, V., Velazquez, A., Yang, L., Yang, Y. C. & Barik, S. (1997).** Transcriptional induction of multiple cytokines by human respiratory syncytial virus requires activation of NF- κ B and is inhibited by sodium salicylate and aspirin. *Virology* **232**, 369–378.
- Chambers, T. J., Hahn, C. S., Galler, R. & Rice, C. M. (1990).** Flavivirus

- genome organization, expression, and replication. *Annual Review of Microbiology* **44**, 649–688.
- Chang, M. F., Chen, C. J. & Chang, S. C. (1994).** Mutational analysis of delta antigen: effect on assembly and replication of hepatitis delta virus. *Journal of Virology* **68**, 646–653.
- Chaturvedi, U. C., Mathur, A., Tandon, P., Natu, S. M., Rajvanshi, S. & Tandon, H. O. (1979).** Variable effect on peripheral blood leucocytes during JE virus infection of man. *Clinical and Experimental Immunology* **38**, 492–498.
- Chen, C. J., Kuo, M. D., Chien, L. J., Hsu, S. L., Wang, Y. M. & Lin, J. H. (1997).** RNA–protein interactions: involvement of NS3, NS5, and 3' noncoding regions of Japanese encephalitis virus genomic RNA. *Journal of Virology* **71**, 3466–3473.
- Chen, N., Warner, J. L. & Reiss, C. S. (2000).** NSAID treatment suppresses VSV propagation in mouse CNS. *Virology* **276**, 44–51.
- Cirino, G. (1998).** Multiple controls in inflammation, extracellular and intracellular phospholipase A₂, inducible and constitutive cyclooxygenase, and inducible nitric oxide synthase. *Biochemical Pharmacology* **55**, 105–111.
- Cossarizza, A., Franceschi, C., Monti, D., Salvioli, S., Bellesia, E., Rivabene, R., Biondo, L., Rainaldi, G., Tinari, A. & Malorni, W. (1995).** Protective effect of N-acetylcysteine in tumor necrosis factor- α -induced apoptosis in U937 cells: the role of mitochondria. *Experimental Cell Research* **220**, 232–240.
- Gilman, A. G., Rall, T. W., Nies, A. S. & Taylor, P. (1990).** *The Pharmacological Basis of Therapeutics*, 8th edn. Elmsford, NY: Pergamon Press.
- Glaser, K. B. (1995).** Regulation of phospholipase A₂ enzymes: selective inhibitors and their pharmacological potential. *Advances in Pharmacology* **32**, 31–66.
- Grilli, M., Pizzi, M., Memo, M. & Spano, P. (1996).** Neuroprotection by aspirin and sodium salicylate through blockade of NF- κ B activation. *Science* **274**, 1383–1385.
- Huang, R. T. & Dietsch, E. (1988).** Anti-influenza viral activity of aspirin in cell culture. *New England Journal of Medicine* **319**, 797.
- Jan, J. T., Chen, B. H., Ma, S. H., Liu, C. I., Tsai, H. P., Wu, H. C., Jiang, S. Y., Yang, K. D. & Shaio, M. F. (2000).** Potential Dengue virus-triggered apoptotic pathway in human neuroblastoma cells: arachidonic acid, superoxide anion, and NF- κ B are sequentially involved. *Journal of Virology* **74**, 8680–8691.
- Kalgutkar, A. S., Crews, B. C., Rowlinson, S. W., Garner, C., Seibert, K. & Marnett, L. J. (1998).** Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science* **280**, 1268–1270.
- Karin, M. (1998).** Mitogen-activated protein kinase cascades as regulators of stress responses. *Annals of the New York Academy of Sciences* **851**, 139–146.
- Khanna, N., Agnihotri, M., Mathur, A. & Chaturvedi, U. C. (1991).** Neutrophil chemotactic factor produced by Japanese encephalitis virus stimulated macrophages. *Clinical and Experimental Immunology* **86**, 299–303.
- Kumar, R., Mathur, A., Kumar, A., Sethi, G. D., Sharma, S. & Chaturvedi, U. C. (1990).** Virological investigations of acute encephalopathy in India. *Archives of Disease in Childhood* **65**, 1227–1230.
- Liao, C. L., Lin, Y. L., Wu, B. C., Tsao, C. H., Wang, M. C., Liu, C. I., Huang, Y. L., Chen, J. H., Wang, J. P. & Chen, L. K. (2001).** Salicylates inhibit flavivirus replication independently of blocking nuclear factor kappa B activation. *Journal of Virology* **75**, 7828–7839.
- Lin, Y. L., Huang, Y. L., Ma, S. H., Yeh, C. T., Chiou, S. Y., Chen, L. K. & Liao, C. L. (1997).** Inhibition of Japanese encephalitis virus infection by nitric oxide: antiviral effect of nitric oxide on RNA virus replication. *Journal of Virology* **71**, 5227–5235.
- Lowy, R. J. & Dimitrov, D. S. (1997).** Characterization of influenza virus-induced death of J774.1 macrophages. *Experimental Cell Research* **234**, 249–258.
- Mathur, A., Bharadwaj, M., Kulshreshtha, R., Rawat, S., Jain, A. & Chaturvedi, U. C. (1988).** Immunopathological study of spleen during Japanese encephalitis virus infection in mice. *British Journal of Experimental Pathology* **69**, 423–432.
- Oh-hashii, K., Maruyama, W., Yi, H., Takahashi, T., Naoi, M. & Isobe, K. (1999).** Mitogen-activated protein kinase pathway mediates peroxynitrite-induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells. *Biochemical and Biophysical Research Communications* **263**, 504–509.
- Patrignani, P. (2000).** Nonsteroidal anti-inflammatory drugs, COX-2 and colorectal cancer. *Toxicology Letters* **112/113**, 493–498.
- Peterhans, E. (1997).** Reactive oxygen species and nitric oxide in viral diseases. *Biological Trace Element Research* **56**, 107–116.
- Primache, V., Binda, S., De Benedittis, G. & Barbi, M. (1998).** In vitro activity of acetylsalicylic acid on replication of varicella-zoster virus. *New Microbiologica* **21**, 397–401.
- Raung, S. L., Kuo, M. D., Wang, Y. M. & Chen, C. J. (2001).** Role of reactive oxygen intermediates in Japanese encephalitis virus infection in murine neuroblastoma cells. *Neuroscience Letters* **315**, 9–12.
- Ravi, V., Parida, S., Desai, A., Chandramuki, A., Gourie-Devi, M. & Grau, G. E. (1997).** Correlation of tumor necrosis factor levels in the serum and cerebrospinal fluid with clinical outcome in Japanese encephalitis patients. *Journal of Medical Virology* **51**, 132–136.
- Sagone, A. L., Jr & Husney, R. M. (1987).** Oxidation of salicylates by stimulated granulocytes: evidence that these drugs act as free radical scavengers in biological systems. *Journal of Immunology* **138**, 2177–2183.
- Schwarz, K. B. (1996).** Oxidative stress during viral infection: a review. *Free Radical Biology and Medicine* **21**, 641–649.
- Schweizer, M. & Peterhans, E. (1999).** Oxidative stress in cells infected with bovine viral diarrhoea virus: a crucial step in the induction of apoptosis. *Journal of General Virology* **80**, 1147–1155.
- Schwenger, P., Alpert, D., Skolnik, E. Y. & Vilcek, J. (1998).** Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced I κ B α phosphorylation and degradation. *Molecular and Cellular Biology* **18**, 78–84.
- Segal, R. A. & Greenberg, M. E. (1996).** Intracellular signaling pathways activated by neurotrophic factors. *Annual Review of Neuroscience* **19**, 463–489.
- Singh, A., Kulshreshtha, R. & Mathur, A. (2000).** Secretion of the chemokine interleukin-8 during Japanese encephalitis virus infection. *Journal of Medical Virology* **49**, 607–612.
- Speir, E., Yu, Z. X., Ferrans, V. J., Huang, E. S. & Epstein, S. E. (1998).** Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. *Circulation Research* **83**, 210–216.
- Srivastava, S., Khanna, N., Saxena, S. K., Singh, A., Mathur, A. & Dhole, T. N. (1999).** Degradation of Japanese encephalitis virus by neutrophils. *International Journal of Experimental Pathology* **80**, 17–24.
- Staal, J. T., Roederer, M., Herzenberg, L. A. & Herzenberg, L. A. (1990).** Intracellular thiols regulate activation of nuclear factor B and transcription of human immunodeficiency virus. *Proceedings of the National Academy of Sciences, USA* **87**, 9943–9947.
- Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., Reynolds, I., Klann, E., Angiolieri, M., Johnson, J. & Defranco, D. B.**

(2000). Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *Journal of Biological Chemistry* **275**, 12200–12206.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature* **231**, 232–235.

Verhaegen, S., McGowan, A. J., Brophy, A. R., Fernandes, R. S. & Cotter, T. G. (1995). Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. *Biochemical Pharmacology* **50**, 1021–1029.

Weissmann, G. (1991). Aspirin. *Scientific American* **264**, 84–90.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. & Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326–1331.

Zhu, H., Bannenberg, G. L., Moldeus, P. & Shertzer, H. G. (1994). Oxidation pathways for the intracellular probe 2',7'-dichlorofluorescein. *Archives of Toxicology* **68**, 582–587.

Received 4 January 2002; Accepted 22 March 2002