Antiviral effect of dehydroepiandrosterone on Japanese encephalitis virus infection

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Japanese encephalitis virus (JEV), which causes neurological disorders, completes its life cycle and triggers apoptotic cell death in infected cells. Dehydroepiandrosterone (DHEA), an adrenal-derived steroid, has been implicated in protection against neurotoxicity and protection of animals from viral-induced encephalitis, resulting in an increased survival rate of the animals. Currently, the mechanisms underlying the beneficial effects of DHEA against the virus are largely unknown. In this study, DHEA suppression of JEV replication and virus-induced apoptosis in murine neuroblastoma (N18) cells was investigated. It was found that DHEA suppressed JEV-induced cytopathic effects, JEV-induced apoptotic cell death and JEV propagation in a concentration-dependent manner. Antiviral activity was more efficient in cultures treated with DHEA immediately after viral adsorption compared with that in cultures receiving delayed administration after adsorption or transient exposure before adsorption. JEV-induced cytotoxicity was accompanied by the inactivation of extracellular signal-regulated protein kinase (ERK). Inactivation of ERK by JEV infection was reversed by DHEA. When cells were treated with the ERK inhibitor U0126, DHEA lost its antiviral effect. Activation of ERK by anisomycin mimicked the action of DHEA in suppressing JEV-induced cytotoxicity. DHEA-related compounds, such as its sulfate ester (DHEAS) and pregnenolone, were unable to suppress JEV-induced cytotoxicity and ERK inactivation. The hormone-receptor antagonists ICI 182780 and flutamide failed to abrogate the antiviral effect of DHEA. These findings suggest that the antiviral effect of DHEA is not linked directly to the genomic steroid-receptor pathways and suggest that the signalling pathways of ERK play a role in the antiviral action of DHEA.

INTRODUCTION

Japanese encephalitis virus (JEV), a member of the family Flaviviridae, contains an ~11 kb, single-stranded, positive-sense RNA genome. The genome serves as the only viral mRNA and encodes a large polyprotein, which is subsequently processed by both host and viral proteases into structural and non-structural proteins (Chambers et al., 1990). JEV infection commonly affects children and is a major cause of acute encephalopathy in several parts of South-East Asia (Umenai et al., 1985). Infection by JEV can cause acute encephalitis with a high mortality rate in humans and induce severe cytopathic effect (CPE) in various types of cultured cells (Vaughn & Hoke, 1992). In JEV-infected patients, the presence of JEV antigens has been detected in certain neural cells including neurons, astrocytes, microglia and vascular endothelial cells (Desai et al., 1995). In addition, neurons in the thalamus, other basal ganglia and the brainstem appear to be particularly vulnerable to JEV infection (Johnson et al., 1985; Chambers et al., 1990). Currently, the detailed mechanisms underlying the neurotropic action of JEV are largely unclear. Based on pathophysiological examination, increasing evidence suggests that both neuronal destruction and dysfunction might partly explain the manifestation of Japanese encephalitis. The disruption of neurotransmitter metabolism and over-activation of inflammatory responses appear to play roles in JEV-induced brain damage (Chaturvedi et al., 1979; Mathur et al., 1988; Yamashita et al., 1989; Khanna et al., 1991; Ravi et al., 1997; Chen et al., 2000, 2004; Singh et al., 2000; Raung et al., 2005). Nevertheless, the mechanisms by which JEV directly induces the death of infected neurons remain largely unknown. Chang et al. (1999) proposed that the four small, hydrophobic, non-structural proteins of JEV might contribute in part to virus-induced CPE via alteration in cell-membrane permeability. Although the generation of free radicals and NF-κB-related signalling molecules is linked to virus-induced CPE (Schwarz, 1996; Marianneau et al., 1997), they have not been demonstrated to be involved in JEV-induced neurotoxicity (Liao et al., 2001, 2002; Raung et al., 2001). Additionally, enforced expression of bcl-2 and...
activation of mitogen-activated protein kinase (MAPK) signalling cascades may protect neurons from JEV-induced cell death in cell cultures (Liao et al., 1997, 2001; Chen et al., 2002). Experimental studies have demonstrated the antiviral effects of non-steroidal anti-inflammatory drugs, salicylates and iminosugar derivatives in culture systems (Liao et al., 2001; Chen et al., 2002; Wu et al., 2002). However, there are no specific antiviral therapeutics available for the treatment of JEV infection. Although the antiviral effect of interferon has been demonstrated in certain RNA virus infections, Solomon et al. (2003) were unable to show a beneficial effect in the outcome of patients with Japanese encephalitis.

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS), precursors of sex steroids, are found naturally in the blood as the most abundant circulating steroid hormones in humans. DHEA is a second adrenal-derived steroid and its serum level is known to exhibit an age-related decline (Majewska, 1995; Kroboth et al., 1999). DHEA concentrations are particularly high in the brain, and DHEA and its related steroids can be synthesized de novo by brain neurons and astrocytes (Zwain & Yen, 1999). DHEA is a widely studied hormone with multi-functional properties. It has been reported to produce beneficial effects in cancer, atherosclerosis, obesity, autoimmune diseases, infections, diabetes and ageing (Coleman et al., 1982; Gordon et al., 1988; Loria et al., 1988; Nestler et al., 1988; Rao et al., 1992; Morales et al., 1994; van Vollenhoven et al., 1994; Bradley et al., 1995; Daigle & Carr, 1998). However, these wide-ranging activities of DHEA cannot be explained fully by activation of the androgen or oestrogen receptors. Furthermore, it is unknown whether all of the effects attributed to DHEA are mediated by DHEA or by its metabolites. Recent studies have pointed out alternative mechanisms by which DHEA exerts its biological action via non-steroid hormone receptors (Compagnone & Mellon, 1998). DHEA also exerts multiple effects (e.g. neuroprotection, plasticity and memory enhancing) in the central nervous system, mediated through its non-genomic actions on neurotransmission, inflammation and oxidative stress (Majewska et al., 1990; Compagnone & Mellon, 1998; Kimonides et al., 1998; Wolf & Kirschbaum, 1999; Barger et al., 2000).

The antiviral effects of DHEA-related hormones have been shown to exert an influence on influenza virus, herpes simplex virus (HSV), coxsackievirus B4, Feline immunodeficiency virus (FIV), human immunodeficiency virus (HIV) and West Nile virus (Loria et al., 1988; Ben-Nathan et al., 1991, 1992; Henderson et al., 1992; Bradley et al., 1995; Daigle & Carr, 1998; Padgett et al., 2000). Most studies have been conducted in animals, and the protective mechanisms induced by DHEA administration have been found to require an intact immune system. Although DHEA protects mice against lethal viral encephalitis (Ben-Nathan et al., 1991), little is known about its effect on virus replication in a cell monolayer in the absence of an immune response. On the other hand, clinical studies indicate that the level of DHEA is lower in HIV-seropositive patients than the normal control and decreases continuously as the disease progresses (Jacobson et al., 1991; Mulder et al., 1992; Uozumi et al., 1996). As DHEA is available as a nutritional supplement and has been administered successfully to humans for a number of clinical syndromes (Coleman et al., 1982; Gordon et al., 1988; Loria et al., 1988; Nestler et al., 1988; Rao et al., 1992; Morales et al., 1994; van Vollenhoven et al., 1994; Bradley et al., 1995; Daigle & Carr, 1998), its potential antiviral action is appreciated and merits further study. In the present study, we explored the effect of DHEA on JEV infection in neuroblastoma cells. We found that DHEA suppressed JEV replication and its cytotoxicity via a non-steroid hormone action. The mechanism of its antiviral action was largely by modulation of the MAPK signalling pathway.

METHODS

Virus and cells. A local Taiwanese strain of JEV, NT113, was used for infection of cells (Chen et al., 2004). Virus propagation was carried out in C6/36 cells grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 % fetal bovine serum (FBS). N18, a mouse neuroblastoma cell line, and baby hamster kidney (BHK21) cells were grown in DMEM containing 10 % FBS.

Virus infection and titre determination. To infect cells with JEV, monolayers were first adsorbed with JEV at an m.o.i. of 5 for 1 h at 37 °C. After adsorption, the unbound virus was removed by gentle washing with PBS, pH 7.4. Serum-free medium was added to each plate for further incubation at 37 °C. To determine viral titres, culture medium was harvested and used in a plaque assay (Chen et al., 2002). Briefly, various dilutions were added to 80 % confluent BHK21 cells and incubated at 37 °C for 1 h. After adsorption, cells were washed and overlaid with 1 % agarose (SeaPlaque; FMC BioProducts) containing RPMI 1640 plus 2 % FBS. After incubation for 4 days, cells were fixed with 10 % formaldehyde and stained with 0.5 % crystal violet.

Cytotoxicity assessment. Cytotoxicity, as indicated by cell-membrane integrity, was assessed by measuring the activity of lactate dehydrogenase (LDH) in the culture medium by the colorimetric detection of formazan using an LDH diagnostic kit (Promega). After experiments, supernatants were transferred to a microtitre plate and incubated with reaction mixture at room temperature for 30 min for colour development. A92 was measured by using a spectrophotometer (PowerWaveX 340; Bio-Tek Instruments).

Western blot. Protein extracts (50 µg) were resolved by SDS-PAGE and transferred on to a blotting membrane. The membrane was first incubated with 5 % skimmed milk in PBS for 30 min to reduce non-specific binding. Subsequently, the membrane was incubated with primary antibodies against JEV NS3 (diluted 1:5000), JEV NS5 (1:5000), β-tubulin (1:1000; Sigma), extracellular signal-regulated kinase (ERK) (1:2000; Santa Cruz Biotechnology) and phospho-ERK (1:2000; Santa Cruz Biotechnology) 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. Signals were developed by chemiluminescent detection. The intensity of signals was determined by using a computer image system (IS1000, Alpha Innotech Corporation).
in vitro with Sp6 RNA polymerase in the presence of Bio-11-UTP (Sigma). Signals were developed by chemiluminescent detection. The intensity of signals was determined by using a computer image system (IS1000, Alpha Innotech Corporation).

**Plaque-reduction assays.** Plaque-reduction assays were carried out as reported previously (Shih et al., 2003). BHK21 cells in monolayers were infected at a virus concentration giving approximately 50–100 plaques per monolayer and were compared with the virus control. DHEA was diluted and included in the agar-medium overlay. Plates were incubated at 37°C for 4 days and stained with crystal violet, and the plaques were counted.

**DNA agarose-gel electrophoresis.** DNA gel electrophoresis was performed as reported previously (Chen & Liao, 2003). Cells were lysed in 0.5% Triton X-100, 5 mM Tris/HCl (pH 7.4), 20 mM EDTA at 4°C for 30 min. After centrifugation, supernatants were extracted with phenol/chloroform and precipitated in ethanol. The resultant DNA (10 µg) was separated on a 1.5% agarose gel and stained with ethidium bromide.

**Flow-cytometry assay.** The cell-cycle distribution was analysed by flow cytometry (Chen & Liao, 2002). Briefly, cells were trypsinized, washed with PBS and fixed in 80% ethanol. They were then washed with PBS, incubated with 100 µg RNase ml⁻¹ at 37°C for 30 min, stained with 50 µg propidium iodide ml⁻¹ and analysed on a FACScan flow cytometer. The percentage of cells in different phases of the cell cycle was analysed by using CellFIT software (Becton Dickinson).

**Statistical analysis.** Data were expressed as the mean ± SEM. For comparisons, the statistical significance between means was determined by using one-way ANOVA followed by Dunnett’s t-test. A level of *P*<0.05 was considered statistically significant.

## RESULTS

**DHEA inhibits the cytotoxicity and replication of JEV**

CPE caused by JEV infection in N18 cells became apparent at 32–48 h post-infection (p.i.), concurrent with the period in which the infected cells actively produced large quantities of virus. CPE was apparent in the pattern of cell death observed by morphological changes and the efflux of LDH. The inhibition of JEV-infected CPE by DHEA is shown in Fig. 1. JEV-infected N18 cells exhibited a typical CPE with detached, rounded cell bodies. CPE was suppressed by the addition of DHEA in a concentration-dependent manner.

Fig. 1. Inhibition of JEV-induced CPE. N18 cells were mock-infected or infected with JEV for 32 h. Various concentrations of DHEA were added as indicated after 1 h viral adsorption. CPE was observed by light microscopy. Bar, 100 µm.
There was no obvious cell damage when the mock-infected cell-culture medium contained the same concentration of DHEA (Fig. 1). JEV-induced LDH efflux was inhibited by DHEA in N18 cells. Furthermore, the antiviral activity of DHEA against JEV was also found in non-neuronal BHK21 cells (Fig. 2a). These results indicated that DHEA is able to suppress JEV-induced CPE.

To investigate whether DHEA had an effect on JEV propagation, we treated N18 cells with various concentrations of DHEA after 1 h virus adsorption. Analysis of cellular viral RNA synthesis (Fig. 2b), viral protein expression (Fig. 2c) and infectious virus-particle release (Fig. 2d) revealed that DHEA-treated cells produced less viral protein and infectious virus compared with the control. To confirm the antiviral activity of DHEA, a plaque-reduction assay was carried out. As shown in Fig. 2(e), DHEA inhibited plaque formation in JEV-infected BHK21 cells. Taken together, these results showed that DHEA treatment suppresses JEV replication and propagation in cells and leads to the attenuation of virus-induced cytotoxicity.

**DHEA inhibits JEV-induced apoptosis**

To understand further the mechanism by which DHEA prevented cell death in JEV-infected cells, several experiments were carried out involving apoptosis. DNA extracted from mock-infected N18 cells was found to be intact whereas, in JEV-infected cells, a characteristic internucleosomal ladder appeared. The addition of DHEA decreased the appearance of the apoptotic DNA ladder in a concentration-dependent manner (Fig. 3a). JEV-induced apoptosis was also analysed by flow cytometry. When JEV was added, N18 cells showed disruption of the normal distribution and a prominent new peak in fluorescence (Fig. 3b). This peak, which represented the sub-G0/G1 phase, is characteristic of cells undergoing apoptosis. When DHEA alone was added to N18 cells, no such peak was observed. The JEV-induced alteration in normal distribution and appearance of the sub-G0/G1 phase were decreased by the presence of DHEA (Fig. 3b). These findings indicated that DHEA inhibits JEV-induced apoptosis in N18 cells, including characteristic internucleosomal fragmentation and the appearance of the sub-G0/G1 phase.

**Action kinetics of DHEA against JEV infection**

To understand further the timing of DHEA inhibition of JEV-induced cytotoxicity, we performed a time-course experiment where DHEA was added at different times relative to the JEV challenge (the reference time, T0, was taken as being after 1 h viral adsorption). DHEA was effective in suppressing JEV-induced cytotoxicity when

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**Fig. 2.** DHEA suppresses JEV replication.

(a) After 1 h mock or virus adsorption, N18 and BHK21 cells were cultivated for a further 40 h in the presence of various concentrations of DHEA. Cell damage was assessed by measurement of LDH efflux ($n=3$). (b) After 1 h mock or virus adsorption, N18 cells were cultivated for a further 24 h in medium alone or in the presence of various concentrations of DHEA. Total cellular RNA was isolated and subjected to slot-blot analysis for the detection of JEV RNA and GAPDH RNA. One of three separate experiments is shown. (c) Cellular extracts were isolated and subjected to Western blot analysis for the measurement of viral NS3, NS5 and $\beta$-tubulin. One of three separate experiments is shown. (d) Cultured media were collected and subjected to a plaque assay for determination of the number of infectious virus particles ($n=3$). (e) Analysis of inhibition of plaque formation was conducted in BHK21 cells, as described in Methods. The percentage of plaque formation was relative to the cell control without DHEA treatment ($n=3$). *$P<0.05$ and **$P<0.01$ compared with the control.
added after viral adsorption, even if administrated up to 8 h after adsorption. However, the cytoprotective potency of DHEA was reduced, especially when added 8 h after adsorption. Importantly, the JEV-induced LDH efflux was not inhibited markedly by the transient exposure of DHEA for 2 h before viral adsorption (Fig. 4). These findings suggested that the cytoprotective action of DHEA is associated with continuous exposure and is restricted to a time window after viral adsorption.

**Inhibition of ERK activation reverses the effect of DHEA**

MAPK cascades play an important role in cell growth, differentiation, survival and apoptosis. Recent evidence indicates that the signalling pathways of MAPK exhibit antiviral activity against flaviviruses, including JEV (Liao et al., 2001; Chen et al., 2002). DHEA administration results in rapid phosphorylation of ERK in endothelial cells (Simoncini et al., 2003). Therefore, we wanted to determine whether the antiviral action of DHEA was as a result of modulation of ERK activation. Infection of N18 cells with JEV resulted in cytotoxicity, whereas the efflux of LDH was suppressed by increasing concentrations of DHEA (Fig. 5a). In parallel, also in a concentration-dependent fashion, rapid phosphorylation of ERK was found in N18 cells after exposure to DHEA (Fig. 5b). To assess further the involvement of the ERK signalling pathway in the antiviral action of DHEA, pharmacological agent U0126 was used, which inhibits ERK. Non-toxic concentrations of U0126 abrogated the protective effect of DHEA against JEV-induced cytotoxicity (Fig. 6a). Phosphorylation of ERK was not repressed markedly by the non-toxic concentration of U0126.
U0126, but this compound enhanced the JEV-induced decrease in the phosphorylation of ERK. Moreover, the restoration of phosphorylation of ERK in JEV-infected N18 cells by DHEA was partially ameliorated by U0126 (Fig. 6b). To demonstrate further the impact of ERK activity on the antiviral effect, anisomycin (an MAPK activator; Konishi et al., 2004) was used. Anisomycin reversed JEV-induced downregulation of ERK phosphorylation and protected cells against JEV infection (Fig. 6c and d). These findings suggested a strong association between the antiviral effect of DHEA against JEV and stimulation of ERK activity.

**Effects of DHEA-related metabolites on JEV-induced cytotoxicity**

DHEA is an adrenal steroid that serves as a precursor to sex hormones and is found naturally in the blood in an

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Fig. 5. DHEA activates ERK phosphorylation. (a) After 1 h mock or virus adsorption, N18 cells were cultivated for a further 32 h in medium alone or in the presence of various concentrations of DHEA. Cellular extracts were isolated and subjected to Western blot analysis for the detection of phospho-ERK (pERK) and total ERK. (b) N18 cells were exposed to various concentrations of DHEA for 1 h. Cellular extracts were isolated and subjected to Western blot analysis for the detection of phospho-ERK and total ERK. One of two independent experiments is shown.

Fig. 6. Role of ERK in the antiviral effect of DHEA. (a) After 1 h mock or virus adsorption, N18 cells were cultivated for a further 40 h in the presence of DHEA (100 µM) or U0126 (0.1 µM) or in combination. Cell damage was assessed by measurement of LDH efflux (n=3). (b) After 1 h mock or virus adsorption, N18 cells were cultivated for a further 32 h in the presence of DHEA (100 µM) or U0126 (0.1 µM) or in combination. Western blot analysis against total ERK or phospho-ERK (pERK) was performed in these lysates. One of two separate experiments is shown. (c, d) After 1 h mock or virus adsorption, N18 cells were cultivated in the absence or presence of anisomycin (1 µM). Cell damage (c) was assessed by measurement of LDH efflux at 40 h p.i. (n=3). Western blot analysis (d) against total ERK or pERK was performed in these lysates at 32 h p.i. One of two separate experiments is shown. **P<0.01.
inactive sulfated form, DHEAS (Kroboth et al., 1999). It is unknown whether the antiviral effect attributed to DHEA is mediated by DHEA or by its related metabolites. To answer this question, we investigated the antiviral effect of the related metabolites of DHEA and the action of a selective hormone-receptor antagonist against the antiviral effect of DHEA. Neither DHEAS nor pregnenolone (a precursor of DHEA; Miller, 1998) induced a statistically significant suppression of JEV-induced cytotoxicity. Moreover, the suppressive effect of DHEA on JEV-induced cytotoxicity was not reversed by either ICI 182780 (an oestrogen-receptor antagonist) or flutamide (an androgen-receptor antagonist) (Simoncini et al., 2003) (Fig. 7). As expected, only protective DHEA reversed the JEV-induced decrease in the phosphorylation of ERK. Treatment with either ICI 182780 or flutamide did not antagonize the effect of DHEA on the phosphorylation of ERK (Fig. 7). These findings suggested that the actions of DHEA are not mediated by a conventional androgen or oestrogen receptor.

**DISCUSSION**

DHEA is the precursor of both oestrogenic and androgenic steroids. Unlike DHEA, the sulfated form of DHEA, DHEAS, and its precursor, pregnenolone (Miller, 1998), failed to protect cells from JEV-induced damage (Fig. 7). Neither oestrogen-receptor antagonist ICI 182780 nor androgen-receptor antagonist flutamide (Simoncini et al., 2003) abrogated the protective effect of DHEA against JEV (Fig. 7). In addition, the effective concentration of DHEA was higher (micromolar level) than that required for the action of hormones (nanomolar level). Our current findings suggest that there must be an alternative mechanism by which DHEA exerts its protective effect against JEV-induced cell injury via non-genomic steroid-hormone receptors.

The exact physiological functions of DHEA are not fully known. DHEA is widely used as an inhibitor of glucose-6-phosphate dehydrogenase (G6PD) activity (Yang et al., 2000). Inhibition of G6PD effectively lowers the cellular pools of ribose 5-phosphate and ultimately results in the inhibition of ribonucleotide and deoxyribonucleotide synthesis. This inhibition may also result in a decrease in NADPH levels, a substrate necessary for the membrane-bound flavoprotein NADPH oxidase to generate superoxide anions (Sankarapandi et al., 1998). Therefore, it is possible that the inhibition of JEV replication and JEV-induced cytotoxicity described herein is linked to the depletion of nucleotide substrates and superoxide-generation substrate. DHEA has been shown to exert its cytoprotective effect by ameliorating lipid peroxidation by a mechanism involving inhibition of hydroxyl radical production (Bastianetto et al., 1999). The involvement of NADPH depletion and the resultant suppression of free radicals in the protective effect of DHEA against JEV were primarily excluded by the findings that free-radical scavenger compounds were ineffective at blocking JEV-induced cell death (Raung et al., 2001). Both DHEA and DHEAS are potent inhibitors of G6PD (Gordon et al., 1995). They have been shown to reverse chloroquine resistance in malaria infection via the inhibition of G6PD (Safeukui et al., 2004). We found that DHEAS was unable to suppress JEV replication (Fig. 7), indicating that direct inhibition of G6PD was not essential for the action of DHEA against JEV infection.

DHEA and related metabolites have been shown to exert regulatory effects on the immune system (Lucas et al., 1985). Administration of DHEA has been reported to protect against lethal viral infection, mostly in animals requiring an intact immune system. However, DHEA has also been found capable of suppressing replication of certain type of viruses, such as HIV (Loria et al., 1988; Ben-Nathan et al., 1991, 1992; Henderson et al., 1992; Bradley et al., 1995; Daigle & Carr, 1998; Padgett et al., 2000). DHEA and DHEAS share similar activity in regulating malaria infection (Safeukui et al., 2004). However, only DHEA was able to suppress FIV replication (Bradley et al., 1995). The antiviral effect of androstenediol against HSV required the production of...
interferon (Daigle & Carr, 1998). Therefore, in addition to diverse characteristics of viruses, DHEA seems to have direct physiological effects on some cell populations, leading to suppression of viral replication.

Many viruses are known to manipulate host-signalling machinery to regulate virus replication and host-gene responses. Among them, MAPKs play important roles and their activities could be modulated in responding to virus infection. MAPKs, which consist of ERK, p38 protein kinase and c-Jun N-terminal kinase (JNK), are central components of signal-transduction pathways in the regulation of cell proliferation, differentiation, cytokine production and apoptosis (Karin, 1998). Several virus infections can induce the activation of MAPKs in infected cells and the ERK pathway has been implicated in the regulation of viral gene expression and replication in, for example, HIV, HSV, human cytomegalovirus, influenza virus and coxsackievirus (Jacque et al., 1998; Zachos et al., 1999; Johnson et al., 2001; Pleschka et al., 2001; Luo et al., 2002). Among the signalling molecules, the inhibition of ERK activity often results in loss of cell viability and apoptosis (Wang et al., 1998). JEV infection resulted in a decrease in ERK phosphorylation (Fig. 5) and in cell death (Figs 1 and 2) and apoptosis (Fig. 3). Previous studies have demonstrated that activation of MAPKs exerts a protective effect against JEV replication and its cytotoxicity (Liao et al., 2001; Chen et al., 2002). The activation of ERK can be modulated by DHEA on endothelial cells (Simoncini et al., 2003). As shown in Fig. 5, DHEA stimulation of the phosphorylation of ERK was concentration-dependent. Administration of DHEA reversed the JEV-induced decrease in ERK phosphorylation. This means that the cytoprotective effect of DHEA from JEV infection might be partly explained by the activation of ERK. To verify this assumption further, we found that the reversal effect from ERK inactivation and the antiviral effect of DHEA against JEV could be attenuated by U0126, an inhibitor of ERK (Fig. 6). In addition, direct activation of ERK by anisomycin also markedly suppressed JEV-induced LDH efflux and ERK inactivation (Fig. 6c and d). Inactive agents, such as DHEAS and pregnenolonone, were unable to reverse JEV-induced ERK inactivation (Fig. 7). Consistent with the failure of ICI 182780 and flutamide to block the antiviral effect of DHEA, they were also ineffective at abrogating the action of DHEA on ERK activation (Fig. 7). Taken together, these results further demonstrate the role of ERK in the antiviral action.

Recently, Liao et al. (2001) demonstrated that the antiflavi-virus effect of salicylates was partially reversed by blocking p38 MAPK activation. Chen et al. (2002) also provided evidence showing the involvement of p38 MAPK and ERK in JEV-mediated cytotoxicity, as well as in the antiviral effect of salicylates. Here, we found that JEV-induced cytotoxicity was associated strongly with downregulation of ERK later in the course of infection (Fig. 5). Transient activation of ERK by DHEA before JEV infection did not exhibit the antiviral effect. The delayed administration of DHEA well after viral infection gradually attenuated its antiviral effect (Fig. 4). Despite the suppressive effect of DHEA, JEV infection still killed the infected cells after further cultivation (data not shown). Overall, DHEA treatment prolonged and/or delayed the onset of JEV-induced cytotoxicity rather than inhibiting it completely. An increasing body of evidence suggests that the dynamic balance between branches of the MAPKs regulates 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 trophic 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). However, studies indicate that ERK activation may also play a pathological role in cells by responding to certain insults (Oh-hashi et al., 1999; Stanciu et al., 2000). Although the detailed mechanisms underlying the antiviral effect remain unclear, our current study provides experimental evidence demonstrating the importance of ERK activation on the antiviral effect of DHEA and suggests that the activation of ERK plays a role in host-cell defence against JEV infection.

In addition to the attenuation of cytotoxicity, DHEA ameliorated JEV replication in cells (Fig. 2). Activation of MAPKs by anisomycin also inhibited JEV replication (data not shown). Our findings and other previous reports (Liao et al., 2001; Chen et al., 2002) indicated that activation of MAPK signalling pathways might interfere with JEV replication. The cellular ERK pathway has been implicated in the regulation of viral gene expression and/or replication. The inhibition of ERK activation in visna virus-infected cells reduced expression of virus-specific proteins, resulting in inhibition of virus replication (Barber et al., 2002). Similar inhibition interfered with the nuclear export of viral proteins, leading to a reduction in virus production (Pleschka et al., 2001). In the HIV model, the activation of ERK augmented viral infectivity and replication via direct phosphorylation of viral proteins (Yang & Gabuzda, 1998). In contrast, the antiviral effect of interleukin 1 against Hepatitis C virus was accompanied by an elevation of ERK activity (Zhu & Liu, 2003). We also demonstrated a strong association between inhibition of JEV replication and ERK activation. It is not known at present how JEV replication is regulated by the ERK signalling pathway. Perhaps the activation of ERK initiates an antiviral cascade in the infected cells. Alternatively, ERK activity might modify and inactivate viral and/or host proteins necessary for the replication of JEV. Taken together, the diversity of ERK involvement suggests its importance either in a global virus strategy to enhance the replicative machinery or as a universal host-defence response to infection. The mechanisms involved in the virus specificity are not fully understood, although they are probably due to differences in the signalling pathways involved in the replication of each virus.

In conclusion, JEV infection induced inactivation of ERK later in the course of infection and caused apoptotic cell...
DHEA suppresses JEV propagation


