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# Effects of Dexamethasone and DHEA on the Responses of Rat Cerebral Cortical Astrocytes to Lipopolysaccharide and Antimycin A

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As part of a study on the effects of dexamethasone and dehydroepiandrosterone (DHEA) on the biological roles of astrocytes in brain injury, this study evaluated the effects of dexamethasone and DHEA on the responses of primary cultured rat cortical astrocytes to lipopolysaccharide (LPS) and antimycin A. Dexamethasone decreased spontaneous release of LDH from astrocytes, and the dexamethasone effect was inhibited by DHEA. However, the inhibitory effect of DHEA on the dexamethasone-induced decrease of LDH release was not shown in astrocytes treated with LPS, and antimycin A-induced LDH release was not affected by dexamethasone or DHEA. Unlike dexamethasone, DHEA increased MTT value of astrocytes and also attenuated the antimycin A-induced decrease of MTT value. Glutamine synthetase activity of astrocytes was not affected by DHEA or LPS but increased by dexamethasone, and the dexamethasone-dependent increase was attenuated by DHEA. However, antimycin A markedly decreased glutamine synthetase activity, and the antimycin A effect was not affected by dexamethasone or DHEA. Basal release of [$^3$H]arachidonic acid from astrocytes was moderately increased by LPS and markedly by antimycin A. Dexamethasone inhibited the basal and LPS-dependent releases of [$^3$H]arachidonic acid, but neither dexamethasone nor DHEA affected antimycin A-induced [$^3$H]arachidonic acid release. Basal IL-6 release from astrocytes was not affected by dexamethasone or DHEA but markedly increased by LPS and antimycin A. LPS-induced IL-6 release was attenuated by dexamethasone but was little affected by DHEA, and antimycin A-induced IL-6 release was attenuated by DHEA as well as dexamethasone. At the concentration of dexamethasone and DHEA which does not affect basal NO release from astrocytes, they moderately inhibited LPS-induced NO release but little affected antimycin A-induced decrease of NO release. Taken together, these results suggest that dexamethasone and DHEA, in somewhat different manners, modulate the astrocyte reactivity in brain injuries inhibitorily.

Key Words: Astrocytes, Dexamethasone, Dehydroepiandrosterone, Lipopolysaccharide, Antimycin A, Glutamine synthetase, Arachidonic acid, Interleukin-6, Nitric oxide

## INTRODUCTION

Neuronal loss in brain ischemia has been attributed to the depletion of cellular energy charge and to cellular acidosis due to anaerobic metabolism, defective ion channel regulation (Siesjö et al, 1987), and loss of mitochondrial integrity by oxygen free radicals formed during reoxygenation (McCord, 1985). And many studies show that all types of neuronal injuries are followed by reactive astrocytosis (Norenberg, 1996), which is inevitably accompanied by activation or/and induction of numerous enzymes, such as phospholipases, glutamine synthetase, and inducible nitric oxide synthase (iNOS), and expressions of cytokines, growth factors, extracellular matrix proteins, and cellular adhesion molecules (Norenberg, 1994, 1996; Kim, 1996). By the way, astrocytes have been known to play an important role in maintaining extracellular milieu of neurons and synapses (Eddleston & Mucke,

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1993), to protect neurons against anoxic (Vibulsreth et al, 1987) and excitotoxic injuries (Rosenberg & Aizenman, 1989; Sugiyama et al, 1989), and to promote neuronal repair and functional recovery (Norenberg, 1996).

Lipopolysaccharide (LPS) is an inflammatory signal to lead the transformation of resting astrocytes to reactive astrocytes (Lieberman et al, 1989; Chung & Benveniste, 1990). And antimycin A, an inhibitor of mitochondrial respiratory complex III, can induce failure of aerobic metabolism in cultured astrocytes (Erecinska et al, 1981; Olson et al, 1986).

Glucocorticoids have been shown to inhibit overexpression of glial fibrillary acidic protein (GFAP) (Nichols et al, 1990a, 1990b; Laping et al, 1991), which is the most common indicator of reactive astrocytosis (Eng et al, 1987). Dexamethasone shows inhibitory effects on phospholipase $A_2$ activity (Hirata et al, 1980), interleukin-6 (IL-6) expression (Benveniste et al, 1990), and NOS induction (Simmons & Murphy, 1993; Demerle-Pallardy et al, 1993).

Many steroid compounds have been found in the brain, and most of them are synthesized *de novo* in the brain from cholesterol, mainly by glial cells (Baulieu & Robel, 1990; Robel et al, 1991; Jung-Testas et al, 1994). Particularly, the concentrations of dehydroepiandrosterone (DHEA) and its sulfate form (DHEA-S) remain virtually unchanged in the rat brain after the removal of steroidogenic endocrine glands, so DHEA has been referred to as a neurosteroid (Corpéchot et al, 1981; Baulieu, 1991; Korneyev et al, 1993; Schumacher et al, 1996). In fact, DHEA and DHEA-S were found in the brain at concentrations much higher than those in the plasma, suggesting that they play functional roles in the CNS (Corpéchot et al, 1981), and the neurosteroids affect neuronal excitability (Majewska, 1992; ffrench-Mullen et al, 1994) and glial cells as well (Chvatal & Kettenmann, 1991; Del Cerro et al, 1995; Garcia-Segura et al, 1996). But the effects of neurosteroids on astrocytes and the functional significance of the effects are less obvious. This study was carried out to investigate the effect of DHEA on the responses of LPS-stimulated astrocytes and of astrocytes in a condition of antimycin A-induced hypoxia, in comparison to the effect of dexamethasone.

## METHODS

### *Primary culture of astrocytes and drug treatment*

The preparation and culture of astrocytes were established by a method modified from McCarthy & de Vellis' (1980). Cerebral cortices of neonatal rats (Sprague-Dawley, 2 days old or younger), free from meninges and vessels, were prepared aseptically in cold phosphate-buffered saline without $Ca^{2+}$ (PBS). The cortices were dissociated through mesh and further triturated with a pipette, and then suspended in DMEM containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 IU/ml-100 $\mu$g/ml). The suspended cells were washed and resuspended in DMEM with FBS and penicillin-streptomycin, and cell count and viability were examined. The cells were cultured in humidified 5% $CO_2$ atmosphere at 37°C. The medium was changed after 24 hours and then twice a week, so that the culture cells could reach to confluence. The confluent cultures were shaken (250 rpm) at 37°C for 12 hours. The adherent cells were washed and subcultured after trypsinized with 0.125% trypsin containing 1.3 mM EDTA. Immunoreactivity of the cultured cells to GFAP was more than 95%.

The astrocytes were cultured in DMEM with 10% FBS and penicillin/streptomycin to reach to confluence. And then, the astrocytes were treated with dexamethasone ($10^{-6}$ M; control, 0.1% ethanol) or DHEA ($5\times10^{-5}$ M; control, 0.1% ethanol) 4 hours prior to the treatment with LPS (from *E. coli* 055: B5; 1 $\mu$g/ml; control, PBS) or antimycin A (50 $\mu$g/ml; control, PBS) in DMEM without serum. After 20 hours of the final treatments, the followings were measured.

### *Lactate dehydrogenase (LDH) release assay*

The culture medium (50 $\mu$l) of astrocytes was reacted with equal volume of LDH substrate solution (Promega) at room temperature for 30 min, and the reaction was terminated by the addition of 50 $\mu$l of 1 M acetic acid. Absorbance of the solution was measured at 492 nm (Korzeniewski & Callewaert, 1983; Decker & Lohmann-Matthes, 1988).

### *MTT assay*

The cell culture in 24-well plate was incubated with 1/10 volume of MTT labeling reagent (3-[4,5-

dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT); 5 mg/ml in PBS), at 37°C for 4 hours. And the formazan crystal produced by the activities of dehydrogenases in viable cells was dissolved in 1 ml of solubilizing solution (10% SDS in 0.01 M HCl) at 37°C for 12 hours, and then the absorbance was measured at 600 nm (Mosmann, 1983).

*Assay of glutamine synthetase activity*

Astrocytes were washed twice with PBS and then scrapped and collected to pellets. The cell pellets were lysed and sonicated in 1.5 ml of 10 mM imidazole-HCl containing 0.5 mM EDTA (pH 7.0). The sonicated solution was used for the assay of enzyme activity. The activity of glutamine synthetase was measured using the spectrophotometric method of Miller et al (1978). The solution of enzyme fraction was incubated at 37°C for 30 min in 1 ml of reaction mixture containing 40 mM imidazole-HCl (pH 7.0), 3 mM glutamine, 0.4 mM sodium ADP, 30 mM $MnCl_2$, 20 mM sodium arsenate, and 60 mM hydroxylamine. The reaction was terminated by 2 ml of 40% trichloroacetic acid solution containing 0.6 M $FeNO_3$. The absorbance of $\gamma$-glutamylhydroxamate produced by the reaction was measured at 500 nm.

*Assay of [$^3$H]arachidonic acid release*

Astrocytes cultured in 24-well plates were refed with fresh culture media containing 1 $\mu$Ci/ml [$^3$H]-arachidonic acid (Amersham) and further incubated for 12 hours. At the end of the incorporation of [$^3$H]arachidonic acid into astrocytes, the cells were washed three times with DMEM containing 1 mg/ml bovine serum albumin (fatty-acid free). [$^3$H]arachidonic acid-incorporated astrocytes in culture were treated with dexamethasone or DHEA, and 4 hours later, with LPS or antimycin A. The 24 hour-release of [$^3$H]arachidonic acid into culture media was measured using $\beta$-scintillation counter.

*Assay of IL-6 release*

IL-6 released into the culture media was measured using murine IL-6 ELISA system (R&D Systems). The culture medium of astrocytes was added into a well coated with anti-IL-6 capture antibody, and incubated at room temperature for 2 hours. The wells were washed with PBS-Tween (0.05%) and added with biotinylated anti-IL-6 antibody. At the end of the incubation for 2 hours, the wells were washed and incubated with strepavidin-conjugate of enzyme for 2 hours. Substrate solution was added to the wells and incubated at room temperature for 30 min. Reaction was stopped and the absorbance was measured at 450 nm.

*Assay of nitric oxide (NO) release*

NO production was determined by measuring the accumulated nitrite using Griess reaction. The culture medium of astrocytes was reacted with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) at room temperature for 10 min (light-proof). The absorbance was measured at 520 nm.

## RESULTS

*Effects of dexamethasone and DHEA on the change of cell viability by LPS or antimycin A*

Dexamethasone decreased LDH release from astrocytes, and DHEA increased MTT value in astrocyte

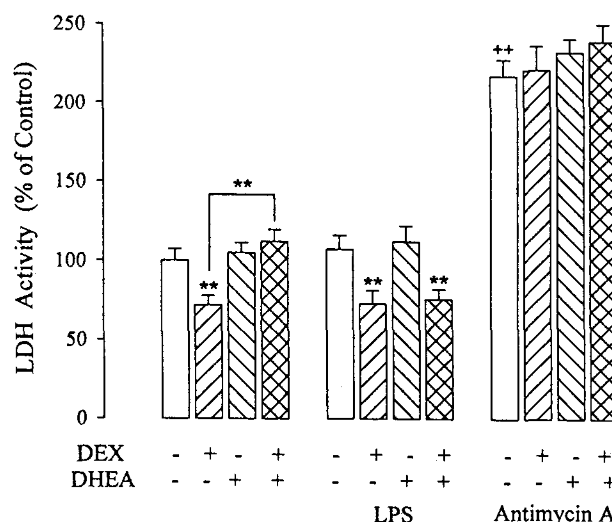


**Fig. 1.** Effects of dexamethasone and DHEA on the change of LDH release from astrocytes treated with LPS or antimycin A. Each bar represents mean±S.E. of 6 independent experiments. ** and * indicate $p<0.05$ and $p<0.1$, respectively, in comparison to the control of each group, and ++ and + indicate $p<0.05$ and $p<0.1$ to the corresponding in the control group.

cultures. DHEA, cotreated with dexamethasone, completely inhibited the dexamethasone-induced decrease of LDH release. In other words, dexamethasone showed protective ability against cytotoxicity, and DHEA showed an ability to increase the mitochondrial activity of astrocytes and to inhibt the anticytotoxic effect of dexamethasone (Fig. 1 & 2).

LPS did not show cytotoxic effect on astrocytes as it hardly affected LDH release and MTT value.

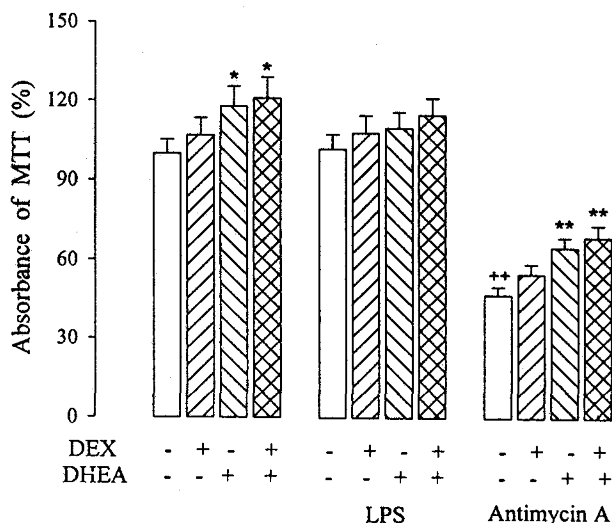


**Fig. 2.** Effects of dexamethasone and DHEA on the change of absorbance of MTT assay in astrocytes treated with LPS or antimycin A.

Dexamethasone also decreased LDH release from astrocytes treated with LPS, and DHEA cotreated with dexamethasone did not affect the dexamethasone-induced decrease of LDH release from astrocytes treated with LPS.

Antimycin A showed evident cytotoxic effect on astrocytes as it increased LDH release and decreased MTT value. Dexamethasone failed to inhibit the antimycin A-induced increase of LDH release, and DHEA partially inhibited the antimycin A-induced decrease of MTT value.

*Effects of dexamethasone and DHEA on the change of glutamine synthetase activity by LPS or antimycin A*

Astrocytes had glutamine synthetase activity of 126.0±7.8 mU/mg protein. Dexamethasone increased glutamine synthetase activity. DHEA showed no effects on glutamine synthetase activity, and partially inhibited the dexamethasone-induced increase of the activity (Fig. 3).

LPS little affected glutamine synthetase activity, and antimycin A decreased glutamine synthetase activity. The effects of dexamethasone and DHEA on LPS-treated astrocytes were similar to their effects on resting astrocytes. But dexamethasone and DHEA showed little effects on antimycin A-induced decrease of glutamine synthetase activity.

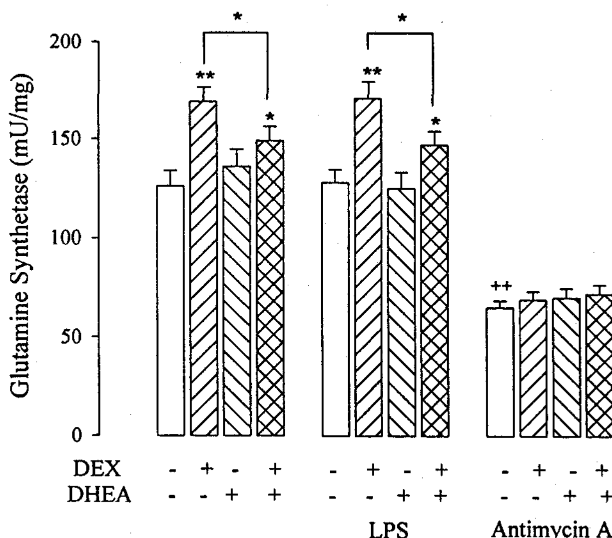


**Fig. 3.** Effects of dexamethasone and DHEA on the change of glutamine synthetase activity of astrocytes treated with LPS or antimycin A.

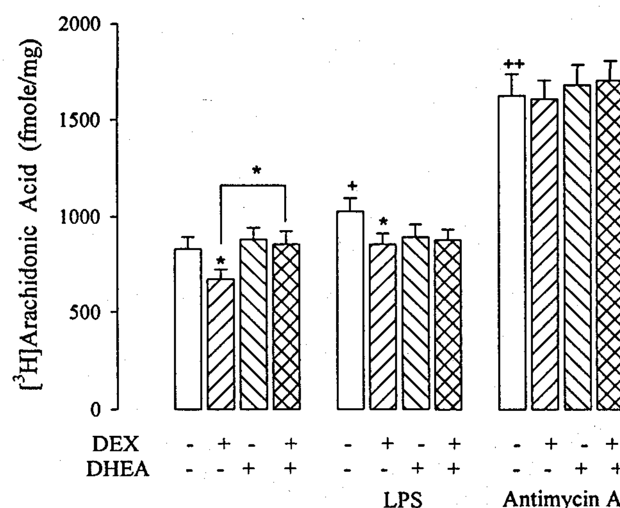


**Fig. 4.** Effects of dexamethasone and DHEA on the change of [$^3$H]arachidonic acid release from astrocytes treated with LPS or antimycin A.

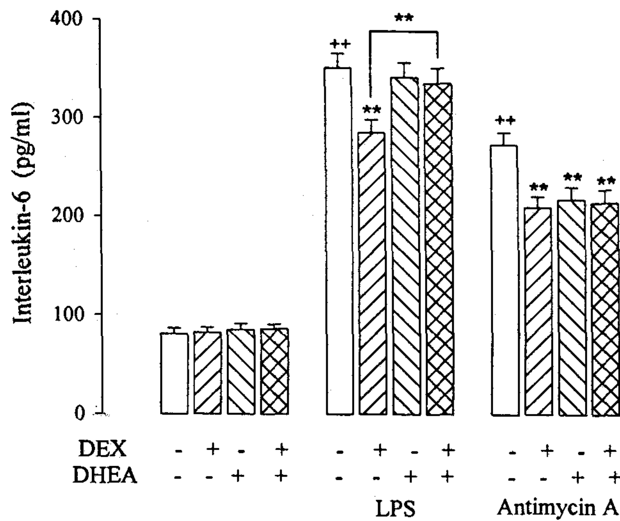


**Fig. 5.** Effects of dexamethasone and DHEA on the change of IL-6 release from astrocytes treated with LPS or antimycin A.

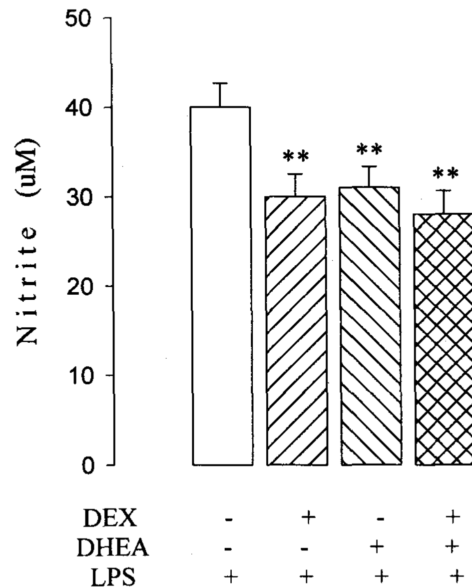


**Fig. 6.** Effects of dexamethasone and DHEA on the LPS-induced release of NO from astrocytes.

*Effects of dexamethasone and DHEA on the change of [$^3$H]arachidonic acid release by LPS or antimycin A*

Dexamethasone slightly decreased basal [$^3$H]arachidonic acid release from astrocytes. DHEA showed little effects on [$^3$H]arachidonic acid release and inhibited the dexamethasone-induced decrease of [$^3$H]-arachidonic acid release (Fig. 4).

LPS and antimycin A increased [$^3$H]arachidonic acid release. Dexamethasone slightly inhibited the LPS-induced increase of [$^3$H]arachidonic acid release, and DHEA did not affect LPS- or antimycin A-induced increase of the release.

*Effects of dexamethasone and DHEA on the change of IL-6 release by LPS or antimycin A*

Dexamethasone and DHEA did not affect the basal IL-6 release from astrocytes (Fig. 5).

LPS increased IL-6 release, and dexamethasone partially inhibited the LPS-induced increase of IL-6 release. DHEA showed no effect on the LPS-induced increase of IL-6 release, and decreased the inhibitory effect of dexamethasone on the LPS-induced increase of IL-6 release. Antimycin A also increased IL-6 release. Dexamethasone and DHEA partially inhibited the antimycin A-induced increase of IL-6 release.

*Effects of dexamethasone and DHEA on the change of NO release by LPS or antimycin A*

Dexamethasone, DHEA, and antimycin A hardly affected NO release from astrocytes (Fig. 6).

LPS induced NO release, and dexamethasone and DHEA partially inhibited the LPS-induced increase of NO release.

## DISCUSSION

High concentration of DHEA is synthesized and presented in central nervous system as well as in circulation, though the precursors and enzymes responsible for its biosynthesis in brain are unknown (Corpéchot et al, 1981; Schumacher et al, 1996). The actions of DHEA have been said to be state-dependency and to buffer the actions of dexamethasone in periphery (Regelson et al, 1990). And interactions of dexamethasone and DHEA in the regulation of proliferation and differentiation of astroglial cells (Jung-Testas et al, 1992; Del Cerro et al, 1995; Crossin et al, 1997) proposed the significance of the interactions of dexamethasone and DHEA concerning the responses of astrocytes to brain injury.

Dexamethasone showed anti-cytotoxic activity (de-

creasing LDH release) in resting astrocytes and LPS-treated astrocytes, but failed to inhibit the high cytotoxic activity of antimycin A. DHEA completely inhibited the anticytotoxic activity of dexamethasone in resting astrocytes, but did not affect the anticytotoxic activity of dexamethasone in LPS-treated astrocytes. DHEA increased the mitochondrial activity (increasing MTT value) in resting astrocytes and also inhibited the decrease of mitochondrial activity in antimycin A-treated cells. But DHEA did not inhibit the increase of LDH release by cytotoxicity of antimycin A. These results showed little relevance to a report that dexamethasone was found to inhibit the proliferation of rat cortical astrocytes in culture and the inhibition was reversed in part by glucocorticoid antagonists including dehydroepiandrosterone (Crossin et al, 1997). But DHEA also showed antagonistic activity to the effect of dexamethasone which inhibited the spontaneous release of LDH in resting astrocytes, and this activity might be associated with the increase of mitochondrial activity by DHEA.

Glutamine synthetase is predominantly localized to astrocytes in the brain, and the increased activity is one of the enzyme activity markers for astrocyte reactivity and associated with differentiation rather than with proliferation of astroglial cells (Martinez-Hernandez et al, 1977; Patel et al, 1983a). The high glutamine synthetase activity in astrocytes can contribute to the control of neuronal injury by providing means to eliminate excessive glutamate. The activity of glutamine synthetase is regulated by glucocorticoids in brain and cultured astrocytes (Patel et al, 1983b; Patel & Hunt, 1985) and transcription of the glutamine synthetase gene is inducible by corticosteroids (O'Banion et al, 1994). The remarkable increase in glutamine synthetase activity in dexamethasone-treated astrocytes suggests that dexamethasone can induce differentiation of astrocytes. Otherwise, the gluamine synthetase activity determines the proportion of glutamate converted to glutamine, so dexamethasone may prepare astrocytes to the increase in glutamate concentration. DHEA also showed an ability to attenuate the increase of glutamine synthetase activity by dexamethasone in this study.

Inhibitory effect of dexamethasone on phospholipase $A_2$ activity (Hirata et al, 1980), on cytokine expression (Benveniste et al, 1990), and on LPS-induction of NOS activity in astrocytes (Simmons & Murphy, 1993; Demerle-Pallardy et al, 1993) is believed to mediate anti-inflammatory and immunosuppressive effects of glucocorticoids in nervous system. Therefore, we investigated the effect of DHEA on these dexamethasone-regulated activities of astrocytes, in addition to the effects on cell viability and on glutamine synthetase activity.

Astrocytes have been known as an important source of eicosanoids in central nervous system (Murphy et al, 1988; Bruner & Murphy, 1990). Various stimuli inducing astrocyte activation also increase arachidonic acid release (Axelrod et al, 1988; Stella et al, 1994), which metabolizes to eicosanoids or moves across the membrane and may be involved in the subsequent autocrine and paracrine effects on astrocytes and neurons (Shimizu & Wolfe, 1990). Release of [$^3$H]arachidonic acid was increased by antimycin A as well as LPS in this study. Dexamethasone showed inhibitory effect on the basal release and the LPS-induced increase, but the increased release by antimycin A seemed to be independent on the glucocorticoid-inhibition (Burgoyne & Morgan, 1990; Gebicke-Haerter et al, 1991). In astrocytes treated with antimycin A, increased release of [$^3$H]arachidonic acid is ascribed to the cell injury in this study.

IL-6 is a pleiotropic cytokine involved in the regulation of inflammation and immune responses (Wong & Clark, 1988; Kishimoto, 1989; Le & Vilcek, 1989). Dexamethasone inhibited IL-6 mRNA expression as well as protein expression in astrocytes (Benveniste et al, 1990). Glucocorticoids generally function as negative regulators for cytokine gene expression (Arya et al, 1984; Lew et al, 1988; Guerne, 1990), and glucocorticoid receptor binding elements have been identified in the 5' flanking upstream region of the IL-6 gene (Tanabe et al, 1988). DHEA showed little effect on LPS-induced increase of IL-6 release and inhibited antimycin A-induced increase in this study. DHEA may show different modes of action on the IL-6 release regulation by LPS or antimycin A. Additionally, DHEA antagonized the inhibitory effect of dexamethasone on LPS-induced increase of IL-6 release.

In brain, iNOS expression are induced in astrocytes (Galea et al, 1992) by exposure to LPS (Tatro et al, 1994). Dexamethasone can be efficient in preventing LPS induction but less so with the cytokine induction of iNOS in astrocytes, and this can be explained by the indirect induction of iNOS by LPS (Simmons & Murphy, 1993). LPS-induced release of cytokines which directly induce iNOS can be inhibited by dexamethasone (Zuckerman et al, 1989). DHEA also inhibited

the LPS-induced increase of NO release from astrocytes, but the effect of DHEA on the expression of iNOS-inducible cytokines remains unclear.

Dexamethasone may not affect the release of biologically active substances from resting astrocytes and the responses of astrocytes to antimycin A which showed cytotoxicity. But dexamethasone can be involved in the process of neuronal injury by the inhibitory effects on the release of arachidonic acid, IL-6 and NO from astrocytes exposed to LPS, and DHEA is supposed to inhibit the changes of astrocyte responses by dexamethasone.

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