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Ethanol-induced Activation of Transcription Factor NF-κB and AP-1 in C6 Glial Cells

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Abstract

In this study, the effects of ethanol and acetaldehyde on DNA binding activities of NF-κB and AP-1 were evaluated in C6 rat glial cells. Both NF-κB and AP-1 are important transcription factors for the expression of various cytokines in glial cells. Our data showed that neither ethanol nor acetaldehyde induced conspicuous cell death of C6 cells at clinically realistic concentrations. When the DNA binding activities of nuclear NF-κB and AP-1 were estimated using electrophoretic mobility shift assay (EMSA), ethanol (0.3%) or acetaldehyde (1 mM) induced transient activation of these transcription factors, which attained peak levels at 4~8 hours and declined to basal levels at 12 hours after treatment. The supershift analysis showed that the increased activities of NF-κB in ethanol/acetaldehyde-treated C6 cells were due to the preferential induction of p65/p50 heterodimer complex. The DNA binding activities of these transcriptional factors decreased below basal levels when cells were cultured with either ethanol or acetaldehyde for 24 hours, and showed the inhibitory effects of chronic ethanol/acetaldehyde treatment on the activities of these transcriptional factors. Our data indicate that either ethanol or acetaldehyde can induce functional changes of glial cells through bi-directional modulation of NF-κB and AP-1 DNA binding activities.

Key words: glia, ethanol, acetaldehyde, NF-κB, AP-1

INTRODUCTION

It has been generally acknowledged that ethanol exerts toxic effects on the central nervous system (CNS). Exposure of the fetus to ethanol in gestation often results in fetal alcohol syndrome associated with impaired maturation of neurons and glial cells. In adults, chronic alcoholism is associated with brain atrophy showing a loss of neurons and reactive astrogliosis, and it is often accompanied by cognitive impairment. In both developing and mature brains, ethanol seems to exert deleterious effects on neurons and glial cells. However the mechanisms mediating ethanol toxicity have not yet been elucidated.

Considering the important role of glial cells in regulating the neuronal micro-environment, functional alterations of glial cells should affect neuronal functions. There are many reports showing that astrocytes, one broad category of glial cells distinguished by their morphology and localization in CNS, are involved in the controlled production of growth factors and cytokines, such as basic fibroblast growth factor, insulin-like growth factor-1, S100, nerve growth factor, interferon-γ (INF-γ), interleukin-1β (IL-1β), IL-3, IL-6, IL-10, tumor necrosis factor α (TNF-α), tumor growth factor β, monocyte/macrophage colony-stimulating factor, and granulocyte/macrophage colony-stimulating factor (GM-CSF) (1,2). These reports indicated that astrocytes can induce the proliferation/degeneration of adjacent cells including neurons and glial cells. These cytokines are important for the modulation of immune responses of CNS. As well, in spite of the traditional view considering CNS as an immunologically privileged organ, recent reports have revealed that CNS is an immuno-competent organ displaying a complex network of various cytokines.

There are two well-known transcription factors that play important roles in the expression of cytokine genes: one is NF-κB and the other is AP-1 (3,4). These transcription factors are also regarded as essential factors for cell proliferation and differentiation. Although the interrelation between these transcription factors and the expression of cytokine genes has been well-established in immune cells, it has been largely ignored in glial cells. Oxidative stresses or reactive oxygen intermediates have been recognized as important activators of these transcription factors, NF-κB and AP-1 (4,5). Also, it is generally assumed that oxygen free radicals and acetaldehyde are implicated in ethanol-induced cytotoxicities in various tissues (6,7). In glial cells, it was demonstrated that hydroxyl radicals and acetaldehyde could be generated from ethanol by cytochrome P4502E1 and alcohol dehydrogenase respectively (8-11).

In this study, we investigated the effects of ethanol on the DNA-binding activities of NF-κB and AP-1 in C6 glial cells. The C6 glial cell line, cloned from a rat glial tumor induced by *N*-nitrosomethylurea (12), is widely used as an experimental model to study the functional changes of glial cells. Our data demonstrated that ethanol and its metabolite, acetaldehyde, induced a transient increase of DNA binding activities of NF-κB and AP-1. Activities of these transcription factors attained their peak levels at 4~8 hours after treatment and returned to the basal levels at 12 hours. These data indicate that either ethanol or acetaldehyde can induce functional

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changes of glial cells through modulation of NF-κB and AP-1 activities.

MATERIALS AND METHODS

Cell culture and media

C6 glial cells were kindly provided by Dr. Y.S. Kim (Seoul National University College of Medicine, Seoul, Korea). C6 glial cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 10 mM Hepes, non-essential amino acids, penicillin (final concentration, 50 units/liter) and streptomycin (final concentration, 50 μg/liter) at 37°C (all from Sigma Chemical Co., St. Louis, MO). When necessary, cultures were incubated in a sealed chamber equilibrated with a designated concentration of ethanol or acetaldehyde.

Fluorescence microscopic assay

Fluorescence microscopic assay was done as described by Hoorens et al. (13) to determine cell viability. The percentage of viable and dead cells was estimated after staining cells with Hoechst 33342 (HO 342; Sigma Chemical Co.) and propidium iodide (PI; Sigma Chemical Co.). HO 342 and PI were added to the medium to a final concentration of 5 μg/ml and cells were examined using inverted fluorescence microscope with ultraviolet excitation at 340~380 nm. Viable and dead cells were identified by their nuclei with blue (HO 342) and red (HO 342 plus PI) fluorescence, respectively. When necessary, floating cells in the medium and cells harvested by trypsinization were collected together, and centrifuged at 800×g for 10 min. After resuspending cells in the medium containing HO 342 and PI, the sample was mounted on a microscopic slide and examined. In each condition and experiment, at least 100 cells were counted. The percentages of viable and dead cells were expressed as means±S.E.M., and all values represented at least three independent experiments.

Fluorescence-activated cell sorting (FACS) analysis

After incubating cells in the medium containing ethanol or acetaldehyde at an appropriate concentration for 24 hours, the percentages of viable and dead cells were estimated on a FACScalibur™ (Becton Dickinson, San Jose, CA) as described by Hamel et al. (14). After collecting the culture medium, cells were rinsed with PBS and detached by trypsinization. The cell suspension was pooled with medium saved from the corresponding culture that contained detached cells. After centrifugation, cells were resuspended in 1 ml of ice-cold PBS containing PI at a concentration of 5 μg/ml. Then, cells were kept at room temperature for 10~15 min and the fluorescence intensity was measured. The data were analyzed by using Cellquest™ software (Becton Dickinson). Percentages of viable and dead cells were expressed as means±S.E.M., and all values represented at least three independent experiments.

Preparation of the nuclear extract

Cells were cultured in a 75 cm^2 tissue culture flask (Falcon, Franklin Lakes, NJ). After washing cells twice with ice-cold PBS, cells were harvested by trypsinization and pelleted by centrifugation at 800×g for 10 min. Then, cells were resuspended in 1 ml of buffer A and pelleted. Buffer A was composed of 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 μg/ml aprotinine and 1 μg/ml leupeptin. Cells were resuspended with one cell volume of buffer A and kept on ice for 10 min. Then, the sample was forced to pass through a 27 gauge needle five times, and centrifuged at 16,000×g in a microcentrifuge for 20 sec. After washing the pellet twice with 500 μl of buffer A, the pellet was suspended in 2/3 cell volume of buffer B and kept on ice for 30 min. Buffer B was composed of 20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol, 1 μg/ml aprotinine and 1 μg/ml leupeptin. After adding 2/3 cell volume of buffer C, the sample was centrifuged immediately at 12,000×g for 15 min at 4°C and the supernatant was stored at -70°C. Buffer C was composed of 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol, 1 μg/ml aprotinine and 1 μg/ml leupeptin. The protein concentration in the extract was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assay (EMSA)

EMSA was done as described in the manufacturer's protocol (Promega, Madison, WI). The sample of 10 μg protein was incubated with 50,000 cpm of a ^{32}P-labeled oligonucleotide containing the consensus sequence for NF-κB or AP-1 in the binding buffer for 30 min at room temperature. The ^{32}P-labeled oligonucleotides were prepared using T4 polynucleotide kinase, 22 bp oligonucleotide containing NF-κB consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3'), or 21 bp oligonucleotide containing AP-1 consensus sequence (5'-CGCTTGATGAGTCAGCCGGAA-3'). The binding buffer was composed of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM EDTA (pH 8.0), 7% glycerol, 0.5 mM DTT, and 0.1 μg/μl poly (dI-dC). In some experiments, unlabeled oligonucleotides containing the consensus sequence for NF-κB, AP-1, or a 22 bp oligonucleotide containing OCT-1 consensus sequence (5-TGTCGAATGCAAATCACTAGAA-3) were added to the extracts before incubation with the labeled oligonucleotides. To identify the protein components involved in the DNA binding activities of NF-κB or AP-1, 0.5 μl of anti-serum specific to NF-κB subunits or AP-1 subunits (all from Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with extract prior to the binding reaction. Samples were electrophoresed on a 6% polyacrylamide gel, and they were subsequently dried and autoradiographed at -70°C overnight.

RESULTS AND DISCUSSION

The cytotoxic effects of ethanol and acetaldehyde on C6 glial cells

C6 cells were cultured in media containing ethanol or acetaldehyde at appropriate concentrations for 24 hours and the percentage of dead cells was estimated. Viable cells could

be distinguished from dead cells by staining nuclei with HO 342 and PI as described in the Materials and Methods. HO 342 freely enters cells with intact membrane as well as cells with damaged membrane, and stains DNA blue. PI, a highly polar dye that is impermeable to cells with intact membrane, stains DNA red. As shown in Fig. 1 and 2, neither ethanol nor acetaldehyde induced conspicuous cell death at concentrations below 2% or 3 mM respectively.

Although it had been reported that chronic ethanol treatment induced transient reactive astrogliosis in the cerebral cortex of rat pups (15), ethanol is generally regarded as an inhibitor against cell proliferation. In *in-vitro* experiments using primary cultured astrocytes, DNA synthesis was inhibited to approximate 75% of the control value in the presence of 0.5% ethanol (16), and ethanol did not induce cell death at a concentration of 200 mM (0.92%) (17). These reports showed resistance of astrocytes to ethanol toxicity, and they coincided with our data. Although the role of acetaldehyde in CNS toxicity still remains unresolved, acetaldehyde and

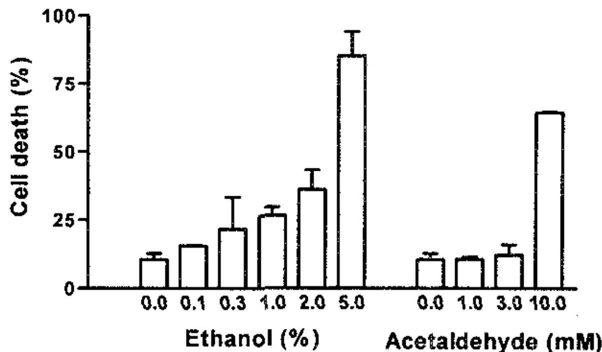

Fig. 1. The cytotoxic effects of ethanol and acetaldehyde on C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for 24 hours, and dead cell numbers were estimated as described in Materials and Methods.

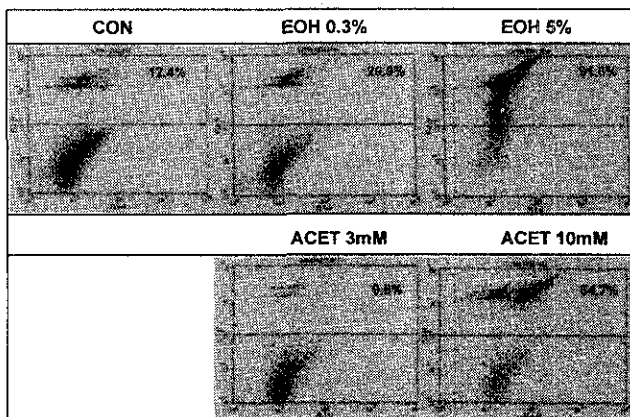

Fig. 2. FACS assay for ethanol or acetaldehyde-induced cell death of C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for 24 hours. After staining cells with HO342 and PI, the percentage of dead cells in the whole cell population was analyzed as described in Materials and Methods. The X-, and Y-axis in each figure show the levels of fluorescence induced by HO342 and PI respectively.

aldehydic products of lipid peroxidation are extraordinarily reactive substances that can form a stable adduct with cellular components to impede normal cellular functions (4). In astrocytes, ethanol can be metabolized to acetaldehyde and subsequently to acetate (8,18). Acetaldehyde originating from liver, the major organ of ethanol metabolism, may play a role in CNS toxicity as well. It was reported that the concentration of acetaldehyde in the brain was about 50% of the value in blood (19). In this study, acetaldehyde did not induce the death of C6 glial cells even at a concentration of 3 mM, which was about one hundred times higher than that in the interstitial fluid of rat brain when serum levels of ethanol ranged from 0.2% to 0.4% (19). Therefore it appears that C6 glial cells are fairly resistant to exogenous acetaldehyde. Similarly, WRL-68 cells derived from human fetal hepatic cells presented 100% viability and no morphological alterations after incubation with 10 mM acetaldehyde for 2 hours, although the proliferative capacity of acetaldehyde-treated cells decreased drastically (20). In addition, the percentage of dead cells were only approximately 22% of the whole cell population when primary cultured rat astrocytes were exposed to acetaldehyde at a concentration of approximately 0.4 mM for 4 days (21). In spite of their inhibitory effects on cell proliferation, these reports and our data suggested that neither ethanol nor acetaldehyde induced the death of glial cells at socially or clinically realistic concentrations.

Transient activation of NF-κB in ethanol-treated C6 glial cells

In monocytes, acute ethanol treatment increased DNA binding activity of NF-κB with a preferential induction of the inhibitory, p50/p50, NF-κB/Rel homodimer and resulted in no induction of p65/p50 heterodimer (22). In this study, EMSA using ^{32}P-labeled oligonucleotides containing NF-κB consensus sequence showed three bands (Fig. 3). The complex formation of ^{32}P-labeled NF-κB oligonucleotides with nuclear proteins was inhibited by adding unlabeled NF-κB oligonucleotides, while it was not inhibited by oligonucleotides containing OCT-1 consensus sequence (Fig. 3). These data indicated that these bands represented the binding activities of nuclear proteins to the NF-κB consensus sequence. Among these bands, the uppermost one represented the NF-κB ac-

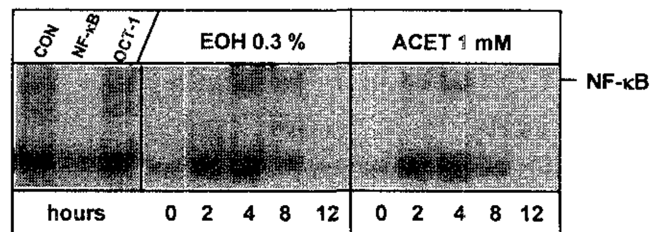

Fig. 3. Time course of NF-κB activation after ethanol/acetaldehyde treatment in C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for indicated time periods and NF-κB DNA binding activities were estimated by EMSA as described in Materials and Methods. EOH, ethanol; ACET, acetaldehyde.

tivity generated by p65/p50 heterodimer as determined by the supershift assay using anti-p65 and anti-p50 antibodies (Fig. 4). In C6 glial cells treated with 0.3% ethanol, DNA binding activity of nuclear NF-κB increased at 4~8 hours and declined to a basal level at 12 hours after treatment (Fig. 3). It appeared that acute ethanol treatment could induce activation of NF-κB at considerably lower concentrations. Figure 5 shows that ethanol at a concentration of 0.005% (1.09 mM) increased the DNA binding activity of NF-κB at 4 hours after treatment.

NF-κB is a transcription factor whose activity is essential to the expression of various cytokine genes. Activation of NF-κB in the cytosol involves phosphorylation of I-κB, and the activated NF-κB is subsequently translocated to the nucleus where it regulates the transcription of target genes.

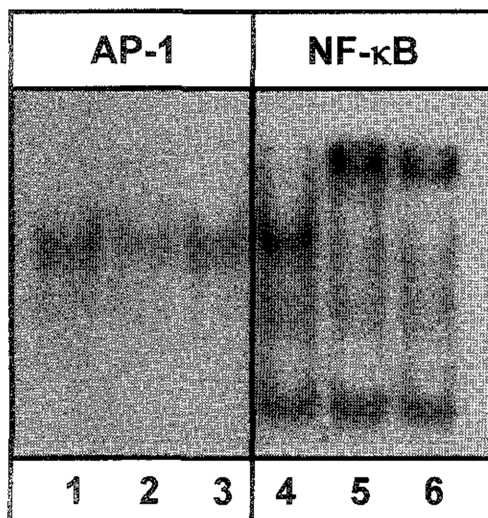

Fig. 4. Supershift analysis of NF-κB and AP-1 in C6 glial cells. An antibody specific to c-Jun (2), c-Fos (3), p65 (5), or p50 (6) was preincubated with nuclear extracts to identify the protein involved in formation of the DNA binding complex as described in Materials and Methods.

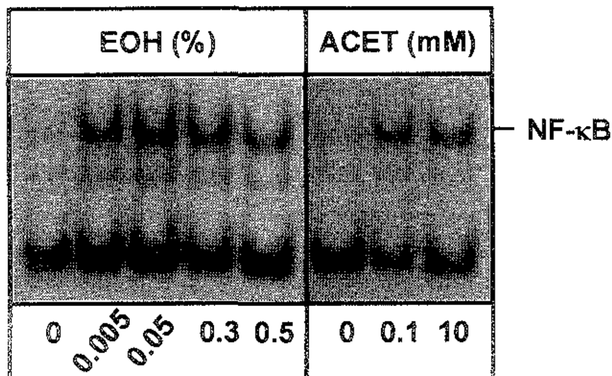

Fig. 5. Dose-dependent effects of ethanol or acetaldehyde on the increase of NF-κB DNA binding activities in C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for 4 hours and NF-κB DNA binding activities were estimated by EMSA as described in Materials and Methods. EOH, ethanol; ACET, acetaldehyde.

Although there are few reports regarding the effects of ethanol on NF-κB activation in glial cells, ethanol has been demonstrated to increase NF-κB activity in various experimental models. In the rat brain, acute administration of ethanol induced the increase of NF-κB activity (23). As well, ethanol induced the activation of NF-κB in vascular smooth muscle cells (11). Oxidative stress is one of well-known activators of NF-κB (24). Reactive oxygen intermediates may be generated through ethanol metabolism (11). In addition, ethanol may increase oxidative stress by altering the fluidity of the mitochondrial membrane and subsequent generation of free radicals and hydrogen peroxide (25). We also observed that ethanol-induced NF-κB activation was prevented by *N*-acetylcystein, an anti-oxidant, in C6 glial cells (data not shown). Considering these reports, it seems probable that oxidative stress generated by ethanol metabolism induced the activation of NF-κB.

In this study, the NF-κB activity decreased below the basal level when cells were cultured in the presence of ethanol for 24 hours (Fig. 6). There is a report showing that the increase of NF-κB activity was retarded in chronic ethanol treatment, which is corresponding to our observation. In rats, NF-κB was activated in the nuclear fraction of the brain after an acute administration of ethanol, while such changes were abolished by chronic ethanol administration (23).

Transient activation of NF-κB in acetaldehyde-treated C6 glial cells

In this study, acetaldehyde at a concentration of 1 mM induced a transient increase of NF-κB activity in C6 glial cells, which was similar to the ethanol-induced effects (Fig. 3). In addition, acetaldehyde seems to increase the DNA binding activities of NF-κB in a dose-dependent manner (Fig. 5). Recent papers have shown that chronic acetaldehyde treatment prevented the activation of NF-κB by inhibiting degradation of I-B in rat liver and Kupffer cells (26,27). Our data also showed that the activity of NF-κB declined to the basal level at 12 hours after acetaldehyde treatment (Fig. 3).

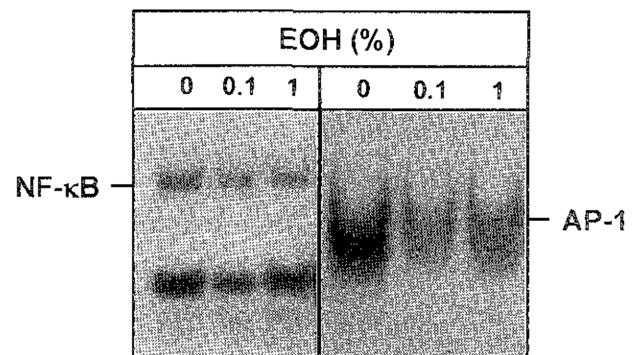

Fig. 6. The effects of ethanol on the DNA binding activities of NF-κB and AP-1 at 24 hours after treatment in C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for 24 hours and DNA binding activities of transcription factors were estimated by EMSA as described in Materials and Methods.

Transient activation of AP-1 in ethanol-treated C6 glial cells

In this study, EMSA using ^{32}P-labeled oligonucleotides containing AP-1 consensus sequence showed only one band (Fig. 7). The complex formation of ^{32}P-labeled AP-1 oligonucleotides with nuclear proteins was inhibited by adding unlabeled AP-1 oligonucleotides, while it was not inhibited by oligonucleotides containing OCT-1 consensus sequence (Fig. 7). These data indicated that this band represented the binding activities of nuclear proteins to the AP-1 consensus sequence. This band was not supershifted by anti-c-Jun and anti-c-Fos antibodies, while preincubation of the nuclear extract with anti-c-Jun antibodies partially abolished the formation of this band (Fig. 4). It was demonstrated that cultured glial cells expressed high basal levels of the AP-1 transcription factor family including c-Jun, Fos-related antigen (Fra), and JunD proteins (28). The protein components concerning ethanol-induced AP-1 activity might be the complex of these proteins.

The ethanol-induced changes of nuclear AP-1 activities were almost identical with those of the NF-κB activities. When cells were treated with 0.3% ethanol, the nuclear AP-1 activity started to increase at 2 hours and attained its peak level at 4 hours. At 12 hours after ethanol treatment, the AP-1 activity returned to a basal level (Fig. 7). The AP-1 activities decreased below basal levels at 24 hours after ethanol treatment (Fig. 6). Fig. 8 shows that ethanol at a concentration of 0.005% (1.09 mM) increased the AP-1 activity at 4 hours after treatment as well. Although acute effects of ethanol on AP-1 activity have not yet been reported, there are several reports that have investigated the chronic effects of ethanol on AP-1 activity. In a recent paper, it was demonstrated that ethanol intake for 15 days did not modulate AP-1 DNA binding activities in the rat brain cortex (29). On the other hand, it was reported that chronic ethanol treatment suppressed the levels of glial fibrillary acidic protein (GFAP) in cultured astrocytes (30). GFAP, the major component of intermediate filaments, is expressed particularly in astrocytes and its expression could be regulated by AP-1 (28,31). These reports and our data suggest that ethanol might induce functional changes of astrocytes through bi-directional modulation of AP-1 DNA binding activities.

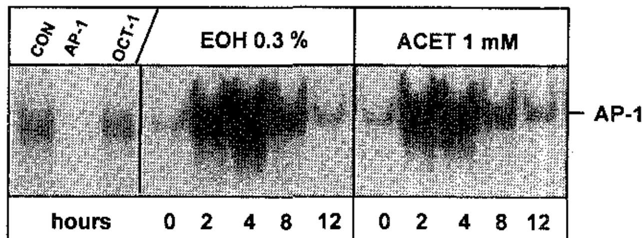

Fig. 7. Time course of AP-1 activation after ethanol/acetaldehyde treatment in C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for indicated time periods and AP-1 DNA binding activities were estimated by EMSA as described in Materials and Methods. EOH, ethanol; ACET, acetaldehyde.

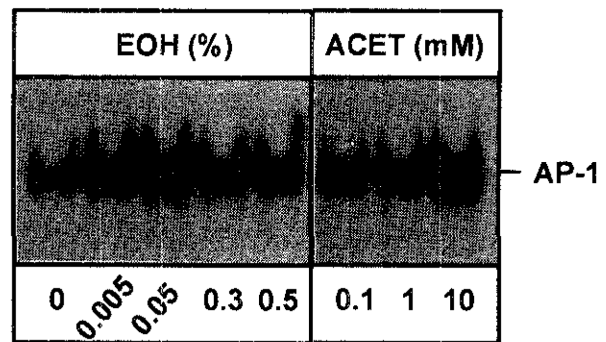

Fig. 8. Dose-dependent effects of ethanol or acetaldehyde on the increase of AP-1 DNA binding activities in C6 glial cells. C6 cells were cultured in the medium containing ethanol or acetaldehyde for 4 hours and AP-1 DNA binding activities were estimated by EMSA as described in Materials and Methods. EOH, ethanol; ACET, acetaldehyde.

Transient activation of AP-1 in acetaldehyde-treated C6 glial cells

There are few reports which have evaluated the effects of acetaldehyde on AP-1 DNA binding activities. In this study, acetaldehyde at a concentration of 1 mM induced a transient increase of AP-1 activity in C6 glial cells, which was similar to the ethanol-induced effects (Fig. 7). In addition, it seemed that acetaldehyde increased the DNA binding activities of AP-1 in a dose- dependent manner (Fig. 8). Our data indicate that acetaldehyde, either derived from circulating blood or converted from ethanol within cells, may induce functional changes of glial cells.

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