Identification of the nuclear transcription factor NFκB in rat brain after in vivo ethanol administration

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Abstract NFκB, a nuclear transcription factor, was induced in the brain nuclear fraction of naive rats after an acute injection of ethanol, 2 g/kg. In contrast, rats which had been chronically alcoholised showed the constitutively active NFκB-like complex only after a further acute dose of ethanol. Hepatic nuclear fractions did not exhibit the specific NFκB-like complex during the first 45 min after acute ethanol injection, beyond that which was normally constitutively present. Such activation of NFκB-like complex in the brains of the naive rats may play an important role in the cellular protective response.

Key words: Nuclear transcription factor; NFκB; Ethanol

1. Introduction

The DNA binding protein nuclear factor, NFκB, is an inducible transcription factor, regulated by translocation from the cytosol to the nucleus in response to extracellular stress factors [1]. NFκB was originally described as a constitutive transcription factor in mature B-cell lines and was subsequently detected in multiple cell types, mainly in an inducible form [2]. NFκB is present in brain tissue, in both the constitutive and inducible forms, with significant amounts of the inducible NFκB forms present in synaptosomes [3]. This may indicate that NFκB has the potential to function as a retrograde messenger, to mediate long-term changes in gene expression following presynaptic stimulation.

Activation of latent NFκB involves phosphorylation of its inhibitor, IκB, which is subsequently proteolytically degraded. Such activation can occur by a variety of factors including exposure to UV, heat shock, viruses, reactive oxygen species such as hydrogen peroxide, modulation of redox state, cytokines such as IL-1 and tumor necrosis factor. NFκB is then translocated to the nucleus where it initiates transcription of target genes (reviewed in [4]).

Excessive intake of ethanol is known to cause extensive perturbations within the cell, which include many of the factors listed above, namely heat shock proteins [5], changes in the secretion of cytokines [6] and tumor necrosis factor [7]. In addition after administration of ethanol in vivo, either acutely or chronically, oxidative stress is enhanced within the brain [8,9] and the liver [10]. Ethanol may alter the fluidity of mitochondrial membranes, with mitochondrial generation of both hydroxyl radicals and hydrogen peroxide [11], while ethanol metabolism within the brain [12] may generate additional radical species contributing to redox changes within the brain. Such alterations could possibly lead to activation of the transcription factor NFκB.

Therefore in these present studies we have investigated whether the transcriptional factor NFκB is present in the isolated nuclear fraction from both brain and liver after administration of an acute dose of ethanol to naive or chronically alcoholised rats.

2. Materials and methods

Male Wistar rats (n=12) were divided into two groups and one group was chronically alcoholised with ethanol vapour within a chamber for 30 days [13]. At the end of this period the rats were removed from the chamber. Rats from each group were injected i.p. with ethanol, 2 g/kg body weight, and then killed at 15 min, 30 min or 45 min post ethanol injection.

The livers and brains were removed at each time point, and a nuclear extract prepared by an adaptation of the method of Spelberg et al. [14]. Homogenates were prepared using a Dounce homogeniser, whole livers, 16% w/v, or whole brains, 10% w/v, in a solution containing 0.32 M sucrose plus 50 mM Tris, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ (TKM buffer), filtered through four layers of cheesecloth and centrifuged at 600×g for 10 min. The pellet was resuspended in 20 ml 2 M sucrose-TKM buffer and homogenised. The homogenate was poured through four layers of cheesecloth and then layered over 2 M sucrose-TKM buffer and centrifuged at 70000×g for 1 h. The pellet containing the nuclear fraction was washed in ice-cold NaCl/P and centrifuged at 15000×g for 15 s prior to resuspension in 200 μl cold buffer A (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM $PhMeSO_2F$, pH 7.9), left on ice for 10 min, vortexed and centrifuged at 15000×g for 30 s. The pellets of nuclei were gently resuspended in 15 ml cold buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (by volume) glycerol, 1 mM dithiothreitol, 0.5 mM $PhMeSO_2F$, pH 7.9) and left for 20 min on ice. After centrifugation (15000×g for 5 min at 4°C) aliquots of the supernatant containing the nuclear protein were rapidly frozen in two aliquots in liquid nitrogen and stored at −80°C. The protein concentrations were assayed prior to the electrophoretic mobility shift assay by BioRad method in one of these aliquots.

2.1. Electrophoretic mobility shift assay

The nuclear fraction containing 20 mg protein was incubated for 30 min at room temperature with 0.2 ng ^{32}P-labelled oligonucleotide probe, 5′-GATCAGGGACTTTCCGCTGGGGACTTTCCAG-3′, 1 mg BSA and 1.25 mg poly(dI-dC), poly(dI-dC) (Pharmacia Biotech Benelux) in buffer (20 mM HEPES/KOH, 75 mM NaCl, 1 mM EDTA, 5% (by volume) glycerol, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) final volume 20 μl.

DNA-protein complexes were then resolved on a non-denaturing 6% (w/v) polyacrylamide gel run for 4 h at 180 V in buffer (2.5 mM Tris, 2.5 mM H_3BO_3, 2 mM EDTA, pH 8.5). The gel was then dried and autoradiographed on a Fuji X-ray film (General Electrics, Antwerp, Belgium). For competition experiments, unlabelled probe (wild type, 5′-GATCAGGGACTTTCCGCTGGGGACTTTCCAG-3′ or mutated 5′-GATCACTCACTTTCCGCTGCTCACTTTCCAG-3′) was added in excess (50×) in buffer.

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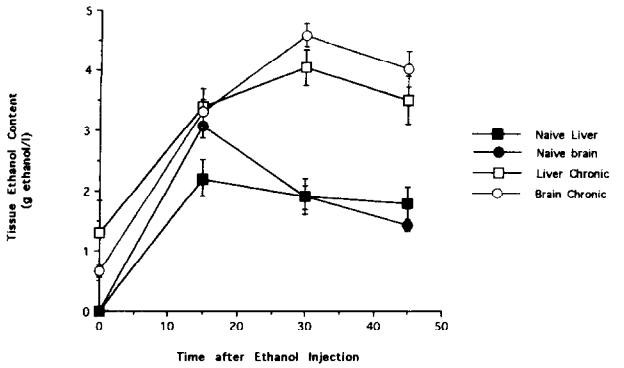


Fig. 1. Concentrations of ethanol in the brains and livers of naive and chronically alcoholised rats, after an acute administration of ethanol, 2 g/kg.

3. Results

The mean concentrations of ethanol in the liver and brain are shown in Fig. 1. In the naive rats, which received an acute ethanol injection of 2 g/kg, the concentration of ethanol was highest at 15 min in both the brain and the liver, after which time it gradually decreased (Fig. 1). In the chronically alcoholised rats there was a more gradual rise in the already elevated liver and brain ethanol content after a further acute ethanol injection, showing a maximum value at 30 min (Fig. 1).

Assay of the nuclear protein by the electromobility shift assay is shown in Figs. 2–4. Within the liver nuclear fractions, a band of extremely low intensity was identifiable which showed little change in its intensity after acute ethanol administration to either naive or chronically alcoholised rats (results not shown). In contrast, the brain nuclear fractions clearly showed the presence of the specific complex after acute ethanol injection, in both naive rats (Fig. 2) and rats chronically administered ethanol (Fig. 3). The intensity of the complex increased only in the naive rats after acute injection of ethanol, 2 g/kg. The identity of this specific complex was confirmed to be NFκB-like by incubating a brain nuclear fraction with excess of the wild probe, after which the complex was not evident in the electromobility shift assay (Fig. 3). Incubation of the sample with excess mutant probe had little effect on the intensity of the NFκB band (Fig. 3). The shift in the brain nuclear fractions was somewhat retarded in comparison to that of the NFκB band observed after incubation of CEM cells with hydrogen peroxide (Fig. 4) and that of the hepatic nuclear fractions.

4. Discussion

In the present study we clearly show that NFκB is activated rapidly in the nuclear fraction of rat brains after acute ethanol administration. Chronically alcoholised rats did not show a comparable activation of NFκB after a further acute ethanol injection, only NFκB constitutively present was identified. In the liver nuclear fractions of either the controls or chronically alcoholised rats no increased activation of NFκB was observed beyond that which was constitutively present.

NFκB is a protein complex that activates the transcription of protective genes involved in inflammation and infection in response to stimuli. A wide range of stimuli have been shown to activate NFκB in vitro, which include heat shock, cytokines such as IL-1 and tumor necrosis factor and hydrogen peroxide. However, there have been few in vivo studies of its activation in either the liver or brain nuclear fractions.

It is clear that ethanol may have an effect upon heat shock gene expression and proteins, particularly within the brain. An ethanol responsive gene, HSC70, a constitutive member of the 70-kDa stress protein family which plays an important role in protein trafficking and coated vesicle processing [15,16], is induced in neuroblastoma and glial cell lines after ethanol incubation. In contrast the liver increased expression of HSP70 mRNA only when severe liver damage was induced by feeding rats fish-oil based ethanol diets [17]. Clearly further studies are needed to identify the role, if any, that heat shock proteins play in the activation and translocation of NFκB to the nucleus.

It is clear that ethanol has an effect on tumor necrosis factor: chronically alcoholised rats fed the Lieber-Decarli diet showed significantly increased levels of TNF in their serum after intravenous endotoxin administration [18], while acute ethanol injection decreased TNF production in response to the endotoxin stimulus [19], the latter effect possibly being exerted in this present study. Ethanol will also alter interleukin levels, IL-1 and IL-6 concentrations in the serum correlating with the extent of liver damage [20]. Since liver damage is not present after an acute ethanol injection, and rats chronically administered ethanol also show little sign of extensive liver damage, we can conclude that such interleukins may not be implicated directly in the activation of NFκB after ethanol injection.

Many of the previous studies of the mode of activation of NFκB have been of cell culture lines, which, after incubation with hydrogen peroxide for a relatively short period of time,

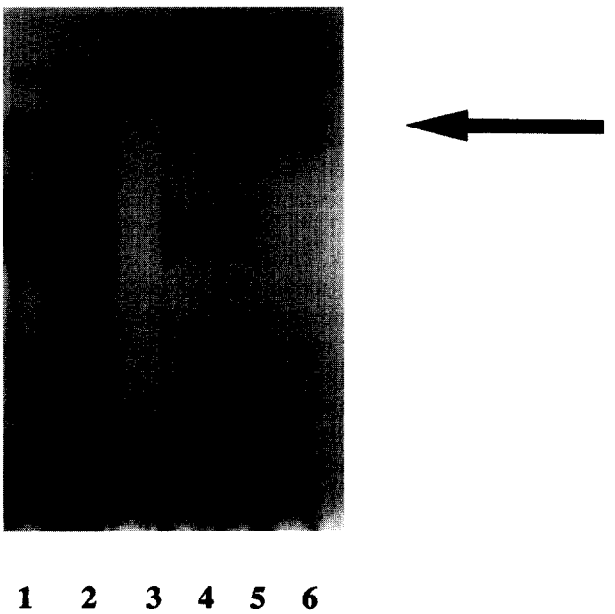
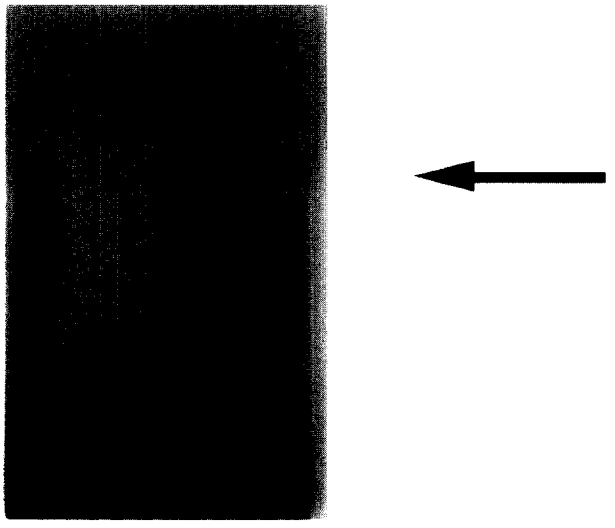


Fig. 2. Identification of NFκB in nuclear pellets isolated from the brains of naive rats at 15, 30 and 45 min after an acute administration of ethanol, 2 g/kg. The arrow marks the position of the specific NFκB complex. Lane 1, 30 min after ethanol; lane 2, 45 min after ethanol; lanes 3 and 4, control; lane 5, 30 min after ethanol; lane 6, 15 min after ethanol.

showed induction of the DNA binding of NFκB [21]. Such activation appears to be a specific event as it occurs at low extracellular concentrations of hydrogen peroxide and other DNA binding proteins do not appear to be affected [21]. Hydrogen peroxide will be produced in vivo in the cell after the superoxide radical has been dismutated by the enzyme superoxide dismutase. SOD is clearly altered after ethanol administration [5,22,23]. Preliminary studies of the activity of SOD in the brain mitochondrial and cytosolic fractions in the present study indicate a transient increase during the first 45 min after ethanol injection which was not apparent in the comparable liver fractions (R.J. Ward, unpublished data). It is clear from cell culture studies that the enzyme SOD plays a pivotal role in the activation of NFκB. Insertion of an extra copy of the Cu/Zn SOD gene into normal fibroblasts and HeLa cells has been shown to increase both lipid peroxidation and expression of NFκB [24,25]

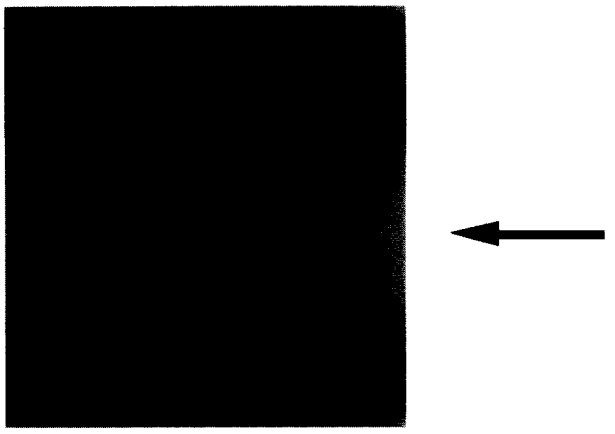
Neuronal cell cultures have been reported to contain constitutively active forms of NFκB in the nucleus. Its presence within neurones indicates that it is likely to participate in normal brain function and to reflect a distinct state of neuronal activity or differentiation [26]. Within the nuclear extracts of the brain we were able to identify bands with NFκB-like activity in both naive and chronically alcoholised rats before injection of ethanol. The intense bands identified after the acute ethanol dose, particularly in the naive rats, showed a higher molecular weight that those identified after administration of hydrogen peroxide to cell cultures. This band is comparable to the BETA band, previously identified in rat brain, with slower gel mobility, with an estimated molecular weight of 125 kDa [27,28].

It was interesting that the chronically alcoholised rats showed less transduction of NFκB than the naive rats despite the higher brain ethanol content. This could be attributable to an increased amounts of catalase within the brain after alcoholisation [6], which would catalyse the degradation of hydrogen peroxide. Insertion of the catalase gene within cells also minimises NFκB expression [29]. Recently it has been reported that stimulation of glutamate receptors, NMDA, kainate and AMPA, activates transcription of NFκB in primary neurone cultures [30]. However, it is unlikely that this has occurred in these present studies, since ethanol, particularly when administered as an acute dose, does not stimulate NMDA receptors [31].



1 2 3 4 5 6

Fig. 3. Identification of NFκB in nuclear pellets isolated from the brains of chronically alcoholised rats at 15, 30 and 45 min after an acute administration of ethanol, 2 g/kg. The arrow marks the position of the specific NFκB complex. Verification of the presence of the NFκB complex was by incubation with excess of wild probe or mutant probe. (See Section 2 for oligonucleotide sequences.) Lane 1, 45 min after ethanol; lane 2, 30 min after ethanol; lane 3, 15 min after ethanol; lane 4, 45 min after ethanol; lane 5, incubation with excess of wild probe; lane 6, incubation with mutant probe.



1 2 3 4 5 6 7

Fig. 4. Activation of NFκB in CEM cells after incubation with H_2O_2. The arrow marks the position of the specific NFκB complex. Lanes 1–3, CEM cells, 1 h, 2 h and 4 h incubation alone; lanes 4–7, CEM cells +H_2O_2 incubation after 1 h, 2 h 3 h and 5 h respectively.

There has been considerable interest in the NFκB in the liver as it possibly plays an important role in the hepatic acute phase response. Kupffer cells have constitutive NFκB binding activity while hepatocytes show no constitutive NFκB but do have inducible NFκB [32]. NFκB binding activity has been demonstrated in nuclear protein extracts of both human hepatoma cell lines and whole rat liver [33,34]. Freedman [32] demonstrated the constitutive presence of NFκB in livers from healthy rats. In these present studies, although confirming the presence of a very low amount of NFκB in the hepatic nuclear fractions, we were unable to demonstrate an increase in its transcription and translocation to the nucleus during the initial 45 min after acute ethanol injection to either naive or chronically alcoholised rats.

The present study has demonstrated a dramatic effect of acute ethanol administration on the activation of NFκB within the brain nuclear fraction of naive rats. The diminished response in the chronically alcoholised rats may indicate some adaptive measures by this group of rat to further insult by the action of ethanol, possibly by the synthesis of catalase. There was no increased activation of NFκB in the livers during the initial 45 min after ethanol administration. The mechanism by which NFκB is activated in the brain has not been clearly elucidated but may be related to alterations in the oxidative tone of the cell or the expression of heat shock proteins. Further studies are clearly needed to ascertain the

mode of activation of this nuclear transcription factor, such that future drug therapy for the prevention of alcohol-induced damage, particularly within the brain, could be directed towards inhibition of this nuclear transcription factor activation.

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