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Increase in catalase activity in developing rat brain cell reaggregation cultures in the presence of ethanol

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Abstract—The aim of this investigation was to study the effects of ethanol on antioxidant enzymes in the developing brain, using reaggregation cultures of fetal rat brain cells as a model. The cultures were grown in the presence of 20 and 40 mM ethanol from day 2 until day 44 of the culture period, corresponding to a period *in vivo* from gestational day 17 to postnatal day 37. The catalase (EC 1.11.1.6) activity was consistently increased at all observation periods, from culture day 11 to day 44, by both doses of ethanol, and an immunoblot showed that the amount of catalase protein was markedly increased. The activities of manganese and copper-zinc superoxide dismutase (EC 1.15.1.1) and glutathione peroxidase (EC 1.11.1.9) were largely unaffected.

It has been suggested that the neurotoxic effects of ethanol might be mediated through the production of free radicals [1]. Several reports indicate that ethanol administration during the fetal and early postnatal periods causes a decrease in superoxide dismutase (SOD,* EC 1.15.1.1) activity, both in cultured astrocytes growing in the presence of 100 mM ethanol [2] and in brains of rats exposed to ethanol either acutely or chronically [3]. Increased lipid peroxidation in the brain after acute ethanol administration has also been reported [4, 5]. The aim of this investigation was to study the effects of clinically relevant doses of ethanol on antioxidant enzymes in the developing brain, using reaggregation cultures of fetal rat brain cells as a model. We have previously shown that the development of the antioxidant enzymes catalase (EC 1.11.1.6), CuZnSOD, MnSOD and glutathione peroxidase (GSHPx, EC 1.11.1.9) in these cultures corresponds well to that *in vivo* [6].

In these cultures, kept as rotation-mediated suspensions, mechanically dissociated fetal rat brain cells spontaneously reassemble into tissue spheroids showing developmental expression and cellular organization of glial and neuronal cells similar to the situation *in vivo* [7, 8]. This culture system offers some advantages over conventional monolayer cultures. It retains much of the complexity of the brain, such as the three-dimensional array of different cell-types supporting each other with growth factors, e.g. nerve growth factor [9], while still being easily manipulated.

Furthermore, it also allows us to study cultures exposed to ethanol during a prolonged period, covering the brain growth spurt, which is considered the most vulnerable period during brain development [10]. This period of rapid growth and myelination occurs during different periods in different animals. In humans, the growth spurt begins in the third trimester and reaches its peak around birth. However, in rats, the comparable growth spurt occurs postnatally, starting a few days after birth and peaking between day 10 and 12 [11, 12]. Thus, with these cultures we have a system of developing rat brain cells in stages corresponding to the brain growth spurt period of the human pregnancy, growing in a serum-free medium with a steady and sufficient supply of nutrients.

Materials and Methods

Reaggregation cultures were prepared according to the method of Honegger *et al.* [7, 8]. Briefly, forebrains (telencephalon + diencephalon) from embryonic (E15-16) Sprague–Dawley rats were dissociated mechanically through nylon nets and the cells were seeded into Erlenmeyer flasks with non-sealing plastic caps and grown in serum-free modified DMEM culture medium under continuous rotation in the incubator. Every third day until 11, 5 mL of medium was changed, and thereafter every 2 days. At day 2 in culture, ethanol was added to give concentrations of 20 and 40 mM, respectively, in the growth medium. The ethanol concentration was kept at these levels throughout the culturing period by addition of freshly prepared ethanol-containing medium at the regular medium changes. The ethanol evaporation under these culturing conditions was less than 5% per day, as determined in separate experiments. Due to the use of ethanol as a solvent for some components added to the medium, the control medium contained 1.5 mM ethanol. Ethanol concentration was measured spectrophotometrically, using the formation of NADH from the oxidation of ethanol by yeast alcohol dehydrogenase [13].

When sampling, cell aggregates were washed twice by centrifugation (300 *g* for 30 sec) in ice-cold 0.25 M sucrose, 10 mM Tris–HCl buffer (pH 7.4), homogenized in 10 mM Tris–HCl (pH 7.4) buffer using a Potter–Elvejhem homogenizer with a Teflon pestle. The homogenates were stored at −70° in aliquots until analysed.

Measurements of protein, DNA, the astrocyte marker glutamine synthetase (EC 6.3.1.2), the oligodendrocyte marker 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP, EC 3.1.4.37), the cholinergic marker choline acetyltransferase (EC 2.3.1.6), and the gabaergic marker glutamate decarboxylase (EC 4.1.1.15), were performed as described previously [6, 9]. Catalase activity was assayed according to the method of Del Rio *et al.* [14], measuring the formation of O_2 from H_2O_2 (33.5 mM) with a Clark-type oxygen electrode, SOD was assayed by the indirect method according to Heikkila [15], by measuring the inhibition of formation of adrenochrome from the spontaneous oxidation of 6-hydroxydopamin, and GSHPx activity was assayed fluorimetrically according to the method of Weiss *et al.* [16], as described previously [6]. Data were evaluated using Kruskal–Wallis' analysis of variance. If significant ($P < 0.05$) differences were indicated by this test, control and ethanol-treated groups were compared using the two-tailed Mann–Whitney U-test.

For immunoblotting, homogenate protein (30 μg/well) was denatured with sodium dodecyl sulfate and separated on a 4–15% Mini-Protean II gradient polyacrylamide gel with molecular weight markers run in parallel. After separation, the proteins were electroblotted onto a nitrocellulose membrane. Thereafter unspecific binding to the membranes was blocked with 3% bovine serum albumin

* Abbreviations: CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; GSHPx, glutathione peroxidase; SOD, superoxide dismutase.

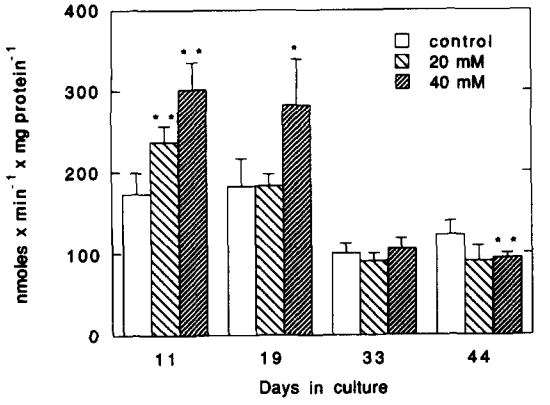

Fig. 1. Activity of CNP, a marker for oligodendrocyte differentiation, in reaggregation cultures exposed to ethanol. Values are presented as mean ± SD, N = 5, asterisks indicate statistically significant differences compared to control (*P < 0.05, **P < 0.01, two-tailed Mann–Whitney U-test).

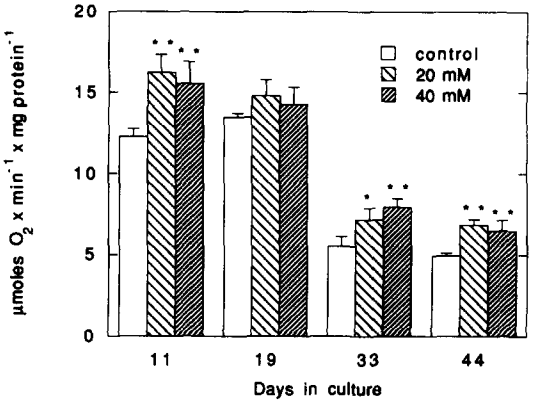

Fig. 2. Catalase activity in reaggregation cultures exposed to ethanol. Values are presented as mean ± SD, N = 5, asterisks indicate statistically significant differences compared to control (*P < 0.05, **P < 0.01, two-tailed Mann–Whitney U-test).

(globulin free) and 0.05% Tween-20. The rabbit anti-rat catalase antiserum (a kind gift of Dr Johan Meijer, Dept Cell Research, Swedish University Agricultural Sciences) was used at a dilution of 1:1000, the biotinylated anti-rabbit antibodies (Amersham, U.K.) at 1:400, and the streptavidin-biotinylated horseradish peroxidase complex (Amersham) at 1:400. As substrates in the colour development reaction 4-chloro-1-naphtol and H_2O_2 were used. The specificity of the anti-catalase antiserum was confirmed by double radial immunodiffusion against purified rat catalase, and by the staining of a single band of an apparent molecular weight of 61 kDa in the immunoblot experiment.

Results

No marked effects were seen on protein or DNA content of the ethanol-treated cultures. A slight, seemingly dose-dependent, but not significant decrease in protein content was observed at later time-points (data not shown).

The cell-specific markers for astrocytes, cholinergic neurons and gabaergic neurons were unaffected by ethanol treatment, but the activity of CNP, the marker enzyme for oligodendrocytes, was increased at day 11 and 19 in culture and slightly decreased at day 44 (Fig. 1).

The catalase activity was consistently increased at both ethanol doses and all sampling times (Fig. 2). No dose–response relationship was observed. The concentration of ethanol present in the assay mixture after dilutions was well below 30 µM, and at this concentration ethanol did not affect the catalase activity when added to control homogenates. The decreased catalase activity in control cultures at day 33 and 44 in culture reflects the normal development in these cultures and in brain [6]. The amount of catalase protein was markedly increased as judged from immunoblots of samples from day 33 in culture (Fig. 3). No bands were detected on an identical blot processed in parallel, using rabbit preimmune serum instead of primary antibody (not shown).

Regarding the activities of SOD (total, Mn and CuZn), and GSHPx, only minor effects were observed (not shown). In a repeated experiment no effects whatsoever of ethanol were found on these enzymes, although the effects on catalase and CNP activities were the same as in the present study (unpublished results).

Discussion

Although ethanol treatment had no effects on SOD and GSHPx activities, the catalase activity was increased both when expressed per mg protein (Fig. 2) or per mg DNA (data not shown). The increase was not due to a direct effect of ethanol on catalase in the assay, in agreement with previous studies [17]. The immunoblot (Fig. 3) shows that this increase in catalase activity is probably due to an increased amount of catalase protein, rather than to an effect on the kinetics of the enzyme. The increased catalase activity could possibly be due to an increased number or accelerated differentiation of oligodendrocytes, as indicated by the increase in CNP activity (Fig. 1). It has been shown that oligodendrocytes contain large amounts of microperoxisomes [18], especially at the onset of myelination [19], and that the catalase activity in the rat brain reaches a maximum during the myelination period [20]. However, whereas the effect of ethanol on CNP at day 11 and 19 seemed to be dose-dependent, the effect on catalase activity was not, and, furthermore, at day 33 and 44, when the CNP activity of ethanol-treated cultures was no longer elevated, the increase in catalase activity still persisted.

It is well known that catalase can oxidize ethanol in the presence of hydrogen peroxide, and catalase-mediated ethanol metabolism has been demonstrated in rat brain homogenates [17, 21, 22]. Since the peak activity of brain catalase occurs during the first postnatal 2 weeks [6], it is possible that the local ethanol metabolism in the brain is higher in newborn than in adult rats. Increased catalase activity has been reported in hearts of ethanol-treated rats [23, 24], an organ which like the brain has low alcohol dehydrogenase activity. It has been argued that the overall production of H_2O_2 in the brain is too low for any significant catalase-mediated ethanol oxidation. It seems, however, that rather high H_2O_2 concentrations can be formed locally, e.g. in peroxisomes and monoamine oxidase-containing nerve terminals.

Increased catalase activity could thus be rather harmful, resulting in a local formation of acetaldehyde, which is a very reactive compound. Acetaldehyde binds covalently to proteins and other macromolecules, and could thus influence their activity and give rise to new antigens, leading to formation of autoimmune antibodies, and further, acetaldehyde is also believed to facilitate lipid

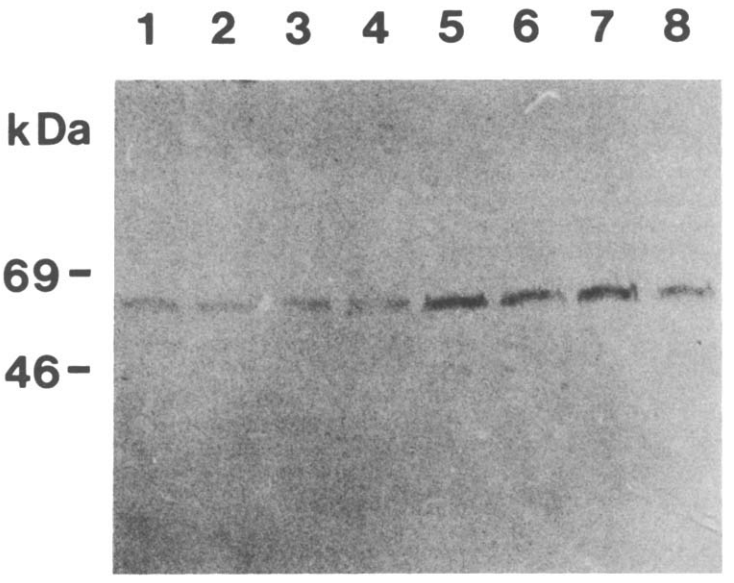

Fig. 3. Immunoblot of catalase, lanes 1–4: samples from different control cultures, lanes 5–8: samples from cultures grown in 40 mM ethanol.

peroxidation by decreasing the glutathione concentration (for a review see Lieber [25]). It is also known that acetaldehyde impairs the metabolism of biogenic amines (dopamine, norepinephrine and serotonin) in the brain by competitive inhibition of aldehyde dehydrogenase at the oxidation of the aldehydes formed from these amines [26, 27]. In fact, inhibition of brain catalase by 3-amino-1,2,4-triazole attenuates or blocks several ethanol-induced behavioural effects. For example, rats pretreated with aminotriazole had shorter periods of narcosis and less lethality [28, 29], less motor depression [30], less ethanol-induced corticosterone release [31], blocked ethanol-induced condition taste aversion [32], and a dose-dependent reduction in ethanol intake.

In summary, we find that chronic administration of ethanol to developing brain tissue results in an increased catalase activity at a period corresponding to the first postnatal weeks in the rat (equivalent to the third trimester in humans). The increased catalase activity and the possible increase in local production of acetaldehyde during this sensitive period could be of importance in the developmental neurotoxicology of ethanol.

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